Drug-Induced Torsade de Pointes Arrhythmias in the Chronic AV Block Dog Are Perpetuated by Focal Activity

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Background—The electrically remodeled canine heart after chronic AV block (CAVB) has a high susceptibility for drug-induced torsade de pointes (TdP) arrhythmias. Although focal mechanisms have been considered for initiation, there is still controversy about whether reentry is the dominant mechanism for perpetuation of TdP. In this animal model with known nonuniform prolongation of repolarization, the mechanism of perpetuation of TdP arrhythmia was explored.

Methods and Results—Seventeen TdP-sensitive CAVB and 10 sinus rhythm (SR) dogs were studied. In 6 animals, 66 needle electrodes were evenly distributed transmurally to record 240 unipolar local electrograms simultaneously. Activation times and activation recovery intervals were determined before and during ibutilide-induced TdP. In 12 CAVB and 9 SR dogs, left ventricular (LV) and right ventricular (RV) epicardial electrograms were recorded with a 208-point multiterminal grid electrode allowing conduction velocity (CV) and ventricular effective refractory period (VERP) measurements. Biopsy specimens were processed for connexin43 (Cx43) expression and collagen content. Ventricular myocytes were isolated to determine sodium current (I\textsubscript{Na}) density and cell dimensions. Computer simulations were used to assess the effects of changes therein. In CAVB, VERP and ARI were increased, whereas CV was unaltered in LV. Transversal but not longitudinal CV was increased in RV. I\textsubscript{Na} was reduced by 37% in LV but unaltered in RV. LV and RV cell size were increased, but collagen and Cx43 content remained unchanged. Simulations showed increase in CV of RV as a consequence of increased cell size at normal I\textsubscript{Na}. Ibutilide increased ARI, ER, and maximal transmural dispersion of ERP (45±25 to 120±65 ms; P<0.05). Twenty-eight of 47 episodes of self-terminating TdP (43±72 beats) were analyzed. The majority (>90%) of beats were focal; reentry was observed only occasionally.

Conclusions—Focal activity is the dominant mechanism involved in perpetuation of ibutilide-induced TdP in CAVB dogs based on detailed 3D mapping. This conclusion is in line with unaltered conduction and documented increase in VERP. (Circ Arrhythm Electrophysiol. 2011;4:566-576.)

Key Words: mechanisms of arrhythmia ▪ conduction ▪ ion channels ▪ long-QT syndrome ▪ torsade de pointes

Since Dessertenne\textsuperscript{1} initially described the torsade de pointes (TdP) arrhythmia in 1966, identification of the arrhythmogenic mechanisms involved in this polymorphic ventricular tachycardia has been the subject of great interest. Especially, the etiology of the acquired, including drug-induced, form of TdP has been the focus of attention. Both in vivo and in vitro studies have revealed that the initiation of TdP was based on early afterdepolarization (EAD)-dependent ectopic activity.\textsuperscript{2-9} The mechanism underlying the continuation of TdP is, however, still under debate. Some investigators claim that reentry is the mechanism of perpetuation because spatial dispersion of repolarization forms the substrate for conduction block,\textsuperscript{2,4-6,10,11} whereas others demonstrate a focal mechanism for the perpetuation of TdP linked to prolonged repolarization because of ventricular remodeling.\textsuperscript{3,7-9} Therefore, we examined the mechanism...
underlying drug-induced TdP in a highly susceptible arrhythmogenic model: the anesthetized chronic AV block (CAVB) dog. This animal model was previously used by 2 groups with different conclusions about whether the mechanism of perpetuation, reentry versus focal activity, was considered dominant.6,8

Clinical Perspective on p 576

Several conditions favor the initiation of reentry, including slowing in conduction, shortening of refractory periods, and heterogeneities in repolarization times (spatial dispersion). Only the latter has been described to be present in the remodeled heart of the CAVB dog.12,13 Therefore, we assessed the molecular and cellular basis for a possible impairment in conduction velocity (CV) in the CAVB dog. What is more, we assessed whether TdP was caused by reentry, focal activity, or both and determined the various electrophysiological and (immuno)histological parameters related to these different mechanisms.

Methods

For a detailed description of methods, see the online-only Data Supplement.

Animals and Preparations

The study protocol was approved by the Committees for Experiments on Animals in Utrecht and Maastricht, The Netherlands, and was conducted in accordance with the European directive for the protection of vertebrate animals used for experimental and other scientific purposes (86/609/EU). Experiments were performed in 27 anesthetized adult dogs (22 to 36 kg) consisting of 17 CAVB and 10 sinus rhythm (SR) control dogs.

Anesthesia and induction of AVB have been described before.13,14 A standard 6-lead ECG was recorded throughout the experiments. Dogs were allowed 6 weeks of ventricular remodeling (mean, 75±31 days; median, 85.5; range, 112 days), after which the mapping experiments were performed.

Recording of Electrograms and Activation Mapping

Detailed 3D activation mapping was achieved in 6 dogs (in 1 during SR and directly after acute AVB [AAVB] and in 5 CAVB dogs) by inserting 66 needle electrodes perpendicular to the ventricular wall. Twenty-seven needles were inserted in the left ventricular (LV) free wall, and another 27 needles were inserted in the interventricular septum. Twelve needles were inserted in the right ventricular (RV) free wall in planes I and III (Figure 1). Two hundred forty unipolar local electrograms were recorded simultaneously. TdP arrhythmias were induced with the use of the class III drug ibutilide (0.025 mg/kg per 5 minutes). Detailed 3D mapping in the SR/AAVB dog was not used to determine activation patterns but to show that activation...
recovery intervals (ARIs) increased from SR to AAVB and finally became maximal in the CAVB dogs; no statistical comparison between SR and CAVB was intended.

Epipolar extracellular electrograms were recorded with a 208-point multiterminal electrode (16×13 grid, 0.5 mm spacing) as described before.15 LV and right ventricular (RV) recordings were made in SR and during stimulation from the center of the grid. To determine transmural CV, a needle electrode (4 electrode terminals) was inserted into the LV free wall.

**Data Analysis and Definitions**

Unipolar epipolar electrograms were transformed into Laplacian electrograms16 to suppress remote signals. The electrograms were analyzed by a semiautomated computer program.17 ARI was defined as the difference between repolarization and activation time.18 CV parallel (θp) and perpendicular (θv) to fiber direction was determined from paced epipolar activation maps. Transmural CV (θpTML) (perpendicular to epicardium) was calculated from the 3 nonstimulated electrode terminals of the transmural needle electrode.

Dispersion of conduction was assessed using the method described by Lamers et al.19 Maximal dispersion of ARI (ΔTSmax) and maximal LV transmural dispersion of ARI (ΔTSmax) were calculated using the difference measured at each separate needle. Activation patterns were determined by plotting the activation times (ΔV/Δmax) referred to as the time difference to the first activated electrode. An episode of TdP was defined as a sequence of at least 5 consecutive beats with a short coupling interval (250 ms) and a twisting QRS morphology on the surface ECG.

**Hypertrophy and Sodium Current Properties**

Total heart to body weight was determined in 12 CAVB and 9 SR dogs. Single myocytes were enzymatically isolated from the subepicardial layer of the LV and RV, as previously described.20 Properties of the sodium current (INa) were measured in subepicardial cells using a standard whole-cell voltage-clamp technique. Capacitance and series resistance were partially compensated for (60% to 80%). Current amplitude (pA) was normalized to cell capacitance (pF). For adequate voltage control, solutions with low concentrations of 5 mmol/L Na+ were used to reduce INa amplitude.

**Immunohistochemistry and Histology**

Biopsy specimens from the LV and RV free wall were harvested from 5 CAVB and 5 SR dog hearts. Frozen biopsy specimens were sectioned in 10-μm slices for immunolabeling and picrosirius red staining. Tissue sections were incubated with antibodies as reported previously.21 For collagen staining, sections serial to picrosirius red staining. Tissue sections were incubated with antibodies as described previously.22

**Connexin43**

Connexin43 (Cx43) protein content was assessed in 2 ways. First, in immunohistochemically labeled sections, both Cx43 content and Cx43 spatial heterogeneity were assessed. Second, Cx43 protein quantification was assessed in ventricular tissue lysates.

**Quantification of Collagen Content**

From picrosirius red-stained sections, digital photomicrographs were analyzed from up to 9 areas, depending on the size of each section.

**Computer Simulations**

CV (θp and θv) was assessed in a linear strand of 100 longitudinally or transversally coupled subepicardial myocytes as described previously.23

**Statistical Analysis**

Statistical significance of differences was evaluated by unpaired Student t test or repeated measurements analyses (with interaction check), χ² test, ANOVA, and Kruskal-Wallis test. Interaction between treatment (SR versus CAVB) and ventricle (LV versus RV) was absent in all cases. Two-sided P<0.05 was considered statistically significant. All data are expressed as mean±SD, unless stated otherwise.

**Results**

**Cardiac and Cellular Hypertrophy**

Heart weight-to-body weight ratio was significantly increased in CAVB hearts (12.0±1.4 versus 8.8±0.9 g/kg; P<0.01). In controls, LV and RV cell length and width were not statistically different (Table 1). In CAVB, myocyte dimensions were significantly increased (Table 1). On average, cell length in RV increased by 28% and in LV by 15%. Average cell width increased in RV by 30% and in LV by 19%.

**Gradients of Repolarization, Electric Remodeling, and Ibutilide-Induced TdP**

In SR (mean heart rate, 110 beats/min), transmural LV ARIs had a mean value of 165±35 ms. Directly after induction of AAVB (mean heart rate, 45 to 50 beats/min), ARIs increased to 250±20 ms (210 electrode terminals). Regional heterogeneities in ARIs were observed as follows: LV>RV, LV septum>RV septum, LV apex>LV base, and LV transmural (endocardial>epicardial) (Figure 2). In AAVB, ΔTMmax and ΔTSmax significantly amounted to 20±10 and 25±25 ms, respectively. In the remodeled CAVB heart (n=5), values increased to 320±30 (ARI), 45±25 (ΔTMmax), and 45±40 ms (ΔTSmax).

Ibutilide prolonged ventricular repolarization (eg, LV ARI from 320±30 to 485±60 ms, PV<0.05) nonhomogeneously; ΔTMmax and ΔTSmax significantly increased to 120±65 and 70±60 ms, respectively. In addition, multiple ectopic beats and 47 TdP episodes were seen in 5 CAVB dogs. Ectopic beats mainly originated from the LV endocardial layer. The majority (83%) of TdP episodes was self-terminating (Figure 3), with an average duration of 4±5 s and 18±27 beats. Activation maps of 28 TdP episodes containing 313 complexes in total were analyzed in part (n=15) or completely. Activation maps of TdP arrhythmias revealed that only <3% of beats were caused by reentry (Figure 4), whereas the majority of TdP beats had a focal origin (Figure 5). The site of earliest activation shifted from beat to beat, whereas occasional competition was observed between multiple foci (beat 3 in Figure 5). Furthermore, an important role for septum in generation of ectopic beats was not observed (Figure 5).

### Table 1. Cell Size and Collagen Content

<table>
<thead>
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<th>SR (n=4)</th>
<th>CAVB (n=3)</th>
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<tr>
<td></td>
<td>RV</td>
<td>LV</td>
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<tr>
<td>Cell size, μm</td>
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<td></td>
</tr>
<tr>
<td>Length</td>
<td>199±40</td>
<td>192±31</td>
</tr>
<tr>
<td>Width</td>
<td>27±5.3</td>
<td>27±5.3</td>
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<tr>
<td>Collagen, % (n=5)</td>
<td>6.5±1.6</td>
<td>4.4±2.3</td>
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</table>

Data are presented as mean±SD. Myocyte dimensions (cell length and cell width) and interstitial fibrosis in both ventricles of SR and CAVB dog heart. Multiple measurements in 1 dog were first averaged and subsequently subjected to repeated-measures analyses. CAVB indicates chronic AV block; LV, left ventricle; RV, right ventricle; SR, sinus rhythm.

*P<0.05 vs the same ventricle in control.
Conduction Velocity

Typical examples of paced epicardial activation maps are shown in Figure 6. Because not all CAVB dog hearts could be paced at the same cycle length, we studied CV as a function of stimulation cycle length. For that purpose, LV and RV of AAVB dogs and CAVB dogs were paced from the grid electrode at different pacing cycle lengths (online-only Data Supplement Figure 1). It was concluded that CV data of both SR and CAVB hearts can be compared.

In CAVB RV, $\theta_T$ was increased compared to SR RV (Table 2). For LV, $\theta_T$ was not different, whereas $\theta_L$ and $\theta_{TM}$ were increased compared to SR LV.

Figure 2. Electric remodeling in AVB dogs. ARIs in 1 dog in SR (left) plus AAVB (middle) in 1 dog with CAVB (right). Shorter ARIs (200 ms) are in dark red, and longer ARIs (400 ms) are in yellow. CAVB increased ARIs and transneedle dispersion (numbers near arrows indicate transneedle dispersion in milliseconds), although dispersion is far from homogeneous. AAVB, indicates acute AV block; ND indicates not determinable; SR, sinus rhythm. Other abbreviations as in Figure 1.
were similar between SR and CAVB dogs. Ventricular effective refractory period (VERP) was significantly increased in both CAVB LV and CAVB RV compared to controls ($P < 0.05$). None of the conduction parameters were significantly altered after a premature stimulus ($S_2$) at the shortest coupling interval (online-only Data Supplement Table 1). During basic stimulation, dispersion of conduction was not significantly different between SR and AAVB dogs.

Figure 3. Self-terminating TdP in a CAVB dog. Six surface-extremity ECG leads show an ibutilide-induced self-terminating TdP (recorded at 10 mm/s). $R$ indicates the reentry beat depicted in Figure 4, whereas the beats indicated with the bar are the activation mapped beats of Figure 5. TdP indicates torsade de pointes.

Figure 4. Activation mapping revealing a reentrant circuit. A. Activation maps in 5 sections of a CAVB dog heart. Activation times are color coded (numbers are activation times in milliseconds). This reentry beat was seen in the LV free wall by electrodes indicated by the section in the box. B. Enlargement of the planes where reentry occurs, revealing activation times and the route of the reentrant circuit (arrows). The letters correspond with the unipolar electrograms shown in C. Reentry starts subendocardially, gradually moving outward to the epicardium in the direction of the base before turning around and going back to the endocardium of the LV free wall. Along the endocardium, the circuit travels apically before reactivating the subendocardium. C. Unipolar electrograms along the reentrant circuit. Activation travels for 192 ms from points A to K. A is again activated after 218 ms. LV indicates left ventricular. Other abbreviation as in Figure 1.
However, dispersion in conduction was significantly increased in LV of CAVB dogs (Table 2).

**Functional Properties of $I_{Na}$**

In control hearts, $I_{Na}$ was significantly larger in LV than in RV cells; current density at $-30 \text{ mV}$ was $-30 \pm 14 \text{ pA/pF}$ in LV versus $-23 \pm 14 \text{ pA/pF}$ in RV. Only in LV was $I_{Na}$ reduced in CAVB ($P<0.05$) (Figure 7A and 7B). In RV cells, current densities were similar in CAVB and control. Activation and inactivation curves of control and CAVB cells were comparable (Figure 7C and 7D) as were the time courses of recovery from inactivation (Figure 7E).

**Fibrosis and Cx43 Expression**

No significant differences were observed in collagen content in the RV between CAVB and SR (Table 1). Moreover, no significant differences in Cx43 protein Western blot analysis in both ventricles in CAVB and SR were seen (Figure 8A), which was confirmed by immunohistochemical analysis (Figure 8C). Although expression of Cx43 was not affected, remodeling changed its distribution: In both RV and LV of CAVB, Cx43 distribution was significantly more heterogeneous (RV, $20.6 \pm 1.0$ versus $15.4 \pm 1.6$ pixels [$P<0.001$]; LV, $17.1 \pm 1.7$ versus $13.6 \pm 0.7$ pixels [$P=0.005$]) (Figure 8B and 8D).

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**Table 2. Electrophysiological Mapping Data**

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<thead>
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<th>SR ($n=9$)</th>
<th>CAVB ($n=9$)</th>
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<tbody>
<tr>
<td>S$_1$S$_2$ Pacing Protocol</td>
<td>RV</td>
<td>LV</td>
</tr>
<tr>
<td>$\theta_L$, cm/s</td>
<td>51.8±9.4</td>
<td>45.2±7.4</td>
</tr>
<tr>
<td>$\theta_T$, cm/s</td>
<td>22.5±4.5</td>
<td>19.5±3.0</td>
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<tr>
<td>$\theta_{TM}$, cm/s</td>
<td>...</td>
<td>36.6±8.5</td>
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<tr>
<td>VERP, ms</td>
<td>190±12</td>
<td>197±22</td>
</tr>
<tr>
<td>Dispersion of CV, cm/s</td>
<td>1.5±0.25</td>
<td>1.1±0.12</td>
</tr>
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</table>

Data are presented as mean±SD. Conduction parameters during S$_1$S$_2$ and VERP. CV indicates conduction velocity; $\theta_L$, $\theta_T$, and $\theta_{TM}$, CV longitudinal or transversal to fiber direction and transmural, respectively; VERP, ventricular effective refractory period. Other abbreviations as in Table 1.

* $P<0.01$ vs same ventricle in control.
† $P<0.01$ vs complementary ventricle in same group. Repeated-measures analysis except for $\theta_{TM}$, which was unpaired Student t test.
‡ $P<0.05$ vs same ventricle in control.
constant while INa remained virtually the same because the reduction of the CV/H9258measurements. Gap junctional resistivity was derived from I junctions per unit membrane surface did not change. Na immunolabeling studies, we assumed that the number of gap
to mean values based on cell size measurements. Based on the Computer-Simulation Studies
indicates sodium current. Other abbreviations as in Figures 1, 2, and 4.

**Figure 7. Properties of INa.** A. Example of peak INa traces in a control and CAVB cell from the LV. Protocol is shown in the inset. B. Current-voltage relation of peak current density; pooled data of 14 control and 25 CAVB cells from the LV (right) and 11 control and 25 CAVB cells from the RV (left). Data are from 6 CAVB (circles) and 4 control (○) dogs. *P<0.05 (repeated-measures analysis). C. Activation curves derived from current-voltage curves and fitted with a Boltzmann equation. For LV (right), V1/2 was −43±3 mV in CAVB and −43±4 mV in control, and k was −3.3±0.8 mV in CAVB and −3.0±0.9 mV in control. Values for RV (left) were V1/2 = −42±3 mV and k=−3.3±0.6 mV in CAVB and V1/2 =−41±3 mV and k = −3.8±1 mV in control. D. Steady-state inactivation during a test step to −30 mV after a 1-s conditioning step at various potentials. Fitting parameters for LV (left) were V1/2 =−83±3 mV and k=5.2±0.3 mV in CAVB (n=8) and V1/2 =−82±2 mV and k = 4.9±0.2 mV in control (n=9), and for RV (right), they were V1/2 = −83±4 mV and k = 4.9±0.6 mV in CAVB (n=8) and V1/2 =−80±4 mV and k = 4.8±0.3 mV in control (n=7). Parameters for activation and inactivation were not statistically different between ventricles or between SR and CAVB (repeated-measures analysis). E. Recovery from inactivation, using a double-pulse protocol with 5-ms increasing intervals between pulses. Current amplitude during the test pulse was normalized to maxi-
mal current at the first pulse. Time course of recovery was fitted with a single exponential and was not different between CAVB (r=16±4 and 19±6 ms for RV and LV, respectively; n=7) and control (r=17±3 and 17±4 ms for RV and LV, respectively; n=5). INa indicates sodium current. Other abbreviations as in Figures 1, 2, and 4.

**Computer-Simulation Studies**

Computer simulations were run with cell length and width set to mean values based on cell size measurements. Based on the immunolabeling studies, we assumed that the number of gap junctions per unit membrane surface did not change. Na+ channel density, however, was adapted according to INa measurements. Gap junctional resistivity was derived from the experimentally observed ϑL and ϑT in SR and was kept constant while INa density, cell dimensions, or both were changed. Simulations suggested that in the RV, both ϑL and ϑT increased in CAVB because remodeling hardly affected INa but increased cell size. In the LV, however, ϑL and ϑT remained virtually the same because the reduction of the CV caused by INa seems to be counterbalanced by enhanced CV due to the increase in cell size (Table 3).

**Discussion**

One of the most important findings of this study was that except for LV INa, none of the parameters involved in determining conduction (fibrosis and Cx43) were significantly affected in the CAVB dog. Cell size was increased in both ventricles. We have clearly shown that the increase in cell size compensated for the anticipated reduction in CV of the LV. Correspondingly, the increased cell size and unaltered INa in the RV resulted in enhanced CV in the RV. The other important finding was that the arrhythmogenic mechanism for the perpetuation of self-terminating TdP was focal in the majority of mapped beats. Reentry played a minor role because it was only sporadically observed.

**CAVB Dog Model, Conduction Parameters, and CV**

The CAVB dog has been used frequently as a reliable model for drug-induced Tdp.12 A Tdp incidence of >70% after administration of proarrhythmic class III drugs normally was seen.

With multiple simultaneously recorded transmural and epicardial electrograms, the anticipated increase in repolarization parameters (ARI and VERP) with CAVB was confirmed. In addition, more-detailed information about regional repolarization gradients was gathered. Spatial dispersion varies between location, with maximal dispersion seen trans-
murally and transseptally. Repolarization differences were further accentuated after administration of the class III agent ibutilide. Because detailed electrophysiological and (immuno)histological characteristics of conduction in the CAVB dog were lacking, the major determinants of impulse conduction in ventricular myocardium were assessed: (1) $I_{Na}$, (2) tissue architecture (fibrosis and cell size), and (3) cell-to-cell coupling (Cx43).

**Sodium Current**

In the CAVB dog, significant downregulation of delayed rectifier current occurs, which contributes to prolongation
of ventricular repolarization compatible with our observation of an increased VERP and ARI. Sipido et al.24 showed enhanced Na⁺/Ca²⁺ exchange activity and suggested that the increase may facilitate arrhythmias by increasing the propensity to sarcoplastic reticulum Ca²⁺ overload, action potential prolongation, and spontaneous Ca²⁺ release. Up to now, the sodium channel has not been studied in the CAVB dog, although subsarcolemmal [Na] has been reported to be increased.25 The fNa in the RV of the CAVB dog was unaltered but reduced in the LV, whereas kinetic parameters were not changed.

**Cell Size and Fibrosis**

The CAVB dog model has been characterized to have biventricular hypertrophy13 because of an increase in cell size of isolated myocytes,26 which was once more confirmed in the present study. Similarly, in line with a previous study, collagen content of CAVB and control dogs was not different.15

**Connexin43**

To our knowledge, connexin expression in the CAVB dog has not been studied thus far. Our data show that Cx43 expression was not altered in both the RV and the LV (Figure 8A and 8C). However, Cx43 protein distribution was more heterogeneous in CAVB in both the RV and the LV (Figure 8B and 8D). In CAVB dogs, increased spatial heterogeneity in Cx43 expression was associated with increased dispersion of conduction, which promotes arrhythmogeneity.27

**Computer Modeling**

Epicardial and transmural CV were not impaired, although fNa in the LV was reduced. We hypothesized that the increase in cell size compensated for the anticipated reduction in CV of the LV. Similarly, because of the increased cell size and unaltered fNa in the RV, CV in the RV should be enhanced. This was exactly what we found in the model of simulated strands of mammalian ventricular cells (Luo-Rudy dynamic model). Indeed, our data show that although fNa was reduced, epicardial CV was higher in CAVB hearts than in controls.

**Mapping TdP**

The mechanism of TdP perpetuation still is not clear. In a recent review,28 2 possible mechanisms have been suggested: focal activity due to EAD-dependent triggered activity and reentry. Both arrhythmogenic mechanisms have been documented in 3D mapping studies.2–11 The current study cannot provide a clear-cut answer to explain these discrepancies. Intrinsic differences between the models used seem a likely explanation. Wherein the first mapping studies methodological differences were quite evident, most recent studies had controversial findings while making use of the same CAVB dog model. Kozhevnikov et al.6 related arrhythmia vulnerability to dofetilide increase in transmural dispersion of repolarization. In contrast, Schreiner et al.8 linked perpetuation of TdP to focal activity with centrifugal spread of activation in 8 of 10 episodes, whereas in the 2 other episodes, reentry was responsible, but an increased spatial dispersion of VERP after almokalant, also a class III agent, was not observed. Moreover, we focused on the initial phase of self-terminating TdP episodes because in later phases of TdP arrhythmia, deterioration in ventricular fibrillation might occur, and reentry might be expected to be the dominant mechanism then. This observation also may explain why others have reported on both mechanisms in this same model. The present study, however, provides solid evidence that focal activity may be the sole mechanism in the perpetuation of early phases of TdP in this model.

In our laboratory, all 3 antiarrhythmic drugs (dofetilide, almokalant, and ibutilide) have been studied. With regard to TdP incidence, TdP burden, TdP duration, or number of defibrillations, no differences existed among the 3 drugs (online-only Data Supplement Table 2).

Perpetuation of reentry is believed to be promoted by reduced CV and a short refractory period. Initiation of reentry is promoted by functional conduction block, which can be caused by either spatial dispersion of repolarization, heterogeneity in conduction parameters, or combinations thereof.

In the present study, there was no impairment in CV or a shortening in VERP and ARI. CV and VERP were affected in the opposite direction, likely excluding a major involvement of reentry in perpetuation of TdP. Both ectopic activity and spatial dispersion of repolarization were, however, abundantly present in the CAVB dog, especially after administration of ibutilide. Careful 3D activation mapping revealed that the majority of ibutilide-induced TdPs were initiated and perpetuated by a nonreentrant (focal activity) mechanism. Most frequently starting in the LV endocardium, activation was radially spread throughout both ventricles. From beat to beat, foci alternate or compete with one another (Figure 5). Additionally, the septum played no important role in generating ectopic beats (Figure 5).

Next to spatial dispersion of repolarization, dispersion in conduction (Table 2) has been mentioned to increase reentry-related arrhythmia vulnerability.29 Therefore, our observation that there was increased dispersion of conduction and re-
larization in the CAVB dog might well explain the sporadically observed reentrant activation. Fragmentation of electrograms and conduction block also were observed occasionally. Still, the relevance of reentry for continuation of TdP is minor. Arguments that microreentry could be part of the story were counteracted by the fact that a large part (40%) of the possible reentrant circuit was consistently missing in the activation sequence. Cycle length of TdP was in the order of 200 ms, of which 110 to 130 ms could be tracked, leaving 70 to 90 ms unaccounted. This time interval would imply that the microreentrant pathway was restricted to <1.5 cm to stay undetected. Our resolution, however, was sufficiently high to make this unlikely.

From these data, it is clear that both mechanisms may play a role. In the present study, we selected TdP episodes that were self-terminating. Because of the acceleration in rate, action potential duration will shorten, heterogeneity will decrease, and EADs will be suppressed. In a minority of TdP episodes, there was degeneration into ventricular fibrillation, which would be an argument in favor of reentry as the culprit mechanism.

Study Limitations

Only short episodes of TdP (<8 s) were recorded and analyzed, so we cannot rule out that in other stages of TdP, reentry plays a more prominent role. Moreover, we have used subepicardial myocytes to better relate the I_{Na} data to the epicardial mapping data. We have not performed I_{Na} measurements on subendocardial myocytes. There may be, however, a relevant difference in I_{Na} strength in the different transmural layers according to recent literature.

Conclusions

The results of the present study indicate that impairment in conduction is unlikely to contribute dominantly to the arrhythmogenic status of the CAVB dog. In contrast, the increased CV is expected to reduce vulnerability for reentry, which is in line with the observation that the arrhythmogenic mechanism for the perpetuation of self-terminating TdP is focal in the majority of mapped beats.

Acknowledgments

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Disclosures

None.

References

The initiation of drug-induced torsade de pointes (TdP) arrhythmias is considered to be based on a focal mechanism: triggering of early afterdepolarizations. The mechanism responsible for TdP continuation is still a matter of debate. Two independent mechanisms are proposed—reentry and early afterdepolarizations—although the combination also has been suggested as being relevant. In this article, we provide evidence that early afterdepolarizations by themselves may be responsible for both the initiation and the continuation of ibutilide-induced TdP. In the chronic AV block dog model, extensive mapping with 66 transmural needles demonstrated that the large majority of beats of self-terminating TdP is caused by early afterdepolarizations. TdP cycle length was in the order of 200 ms, of which 110 ms could be activation mapped. The unaccounted time period in combination with the spatial distribution of the needles (1-cm spacing) suggest sufficient resolution to detect (macro)reentry. Reentry, however, was demonstrated only occasionally. A second reason that makes reentry unlikely is the fact that 2 of its ingredients, (1) slowing of conduction and conduction block and (2) reduced effective refractory period, were altered in the opposite direction in the chronic AV block dog model, whereas spatial dispersion of repolarization was increased. Moreover, the cellular factors relevant for conduction were not changed. The study shows that focal activity is the dominant mechanism involved in perpetuation of ibutilide-induced TdP in chronic AV block dogs.
Drug-Induced Torsade de Pointes Arrhythmias in the Chronic AV Block Dog Are Perpetuated by Focal Activity

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DATA SUPPLEMENT TO

Drug-Induced Torsade de Pointes Arrhythmias in the Chronic AV-Block Dog Are Perpetuated by Focal Activity

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EXTENDED METHODS

1. Animals and preparations

The study protocol was approved by the Committees for Experiments on Animals in Utrecht and Maastricht and was conducted in accordance with the European directive for the protection of vertebrate animals used for experimental and other scientific purposes (86/609/EU). Experiments were performed in 27 anesthetized adult dogs (22 to 36 kg) consisting of 17 chronic complete AV block (CAVB) and 10 sinus rhythm (SR) control dogs.

Anesthesia¹ and induction of AV-block (AVB)² have been described before. A standard 6-lead ECG was recorded throughout the experiments. Dogs were allowed ≥ 6 weeks of ventricular remodeling (mean 75±31 days) after which the mapping experiments were performed.

2. Recording of electrograms and activation mapping

Both ventricles were accessed through a left sided thoracotomy in the 5th intercostal space. In case of TdP mapping, the 4th rib was removed. Whilst the thorax was open a positive end expiratory pressure was used to maintain a proper blood oxygen saturation and pCO₂. Detailed activation mapping was achieved in 6 dogs: in 1 dog during sinus rhythm (SR) and directly after acute AVB and 5 CAVB dogs, by impaling 66 needle electrodes perpendicular to the wall. Twenty seven needles were impaled in the left ventricular free wall (evenly distributed over 5 planes), another 27 needles in the interventricular septum (evenly distributed over the same planes). Twelve needles were impaled in the right ventricular free wall in the planes I and III (Figure 1). The interventricular septum was approached through the right ventricular cavity perpendicular to the septum. 240 Unipolar local electrograms were recorded simultaneously. Needles harbored 4 (for LV and septum) or 2 (for RV) Ag/AgCl electrode terminals with an interelectrode distance of 4 mm. The 66 needles were equally distributed over the 5 planes. A
needle in the pectoral muscle was used as a reference electrode. Needle recordings (Figure 1) were stored for a max of 8 sec using a custom designed software package and amplifier (Biosemi, Amsterdam). TdP arrhythmias were induced with the use of the class III drug Ibutilide (0.025 mg/kg/5’).

Epicardial extracellular electrograms were recorded with a 208 point multi-terminal electrode (16x13 grid, 0.5 mm spacing) as described before. LV and RV recordings were made in SR and during stimulation (1 ms duration, twice stimulation threshold) from the center of the grid. The effective refractory period (VERP), the coupling interval of the shortest premature stimulus that failed to activate the entire heart, was determined. Every sixteenth stimulus was followed by one premature stimulus. Conduction block within the recording area was supposed to occur if activation delay between two adjacent recording sites was >5 ms (conduction velocity <0.06 mm·ms⁻¹).

During epicardial pacing the S₁S₁ cycle length was different in CAVB dogs (both LV and RV S₁S₁ = 783±17 ms) and SR dogs (LV S₁S₁ = 390±7 ms; RV S₁S₁ = 383±9 ms). This was due to the fact that not all CAVB dog hearts could be paced at a cycle length of 390 ms. To address this inconsistency, we evaluated CV as a function of stimulation cycle length. For that purpose, LV and RV of acute AVB dogs (n=4) and chronic AVB dogs (n=3) were paced from the grid electrode at pacing cycle lengths of 800, 600, 400, 350, and 300 ms.

To determine transmural conduction velocity, a needle electrode was impaled into the LV free wall. The needle electrode contained 4 unipolar electrode terminals with 5 mm interelectrode distance. Stimulation was done from the epicardial electrode terminal.

3. Data analysis and definitions

Unipolar epicardial electrograms were transformed into Laplacian electrograms to suppress remote signals. The electrograms were analyzed by a semi automated computer program which determined the activation time as the maximum negative slope in the QRS
(ΔV/Δt_{min}) and the repolarization time as the maximal positive slope in the T-wave (ΔV/Δt_{max}). Activation times of at least 4 consecutive electrode terminals along lines perpendicular to intersecting isochronal lines were used to determine CVs. The activation recovery interval (ARI) was defined as the difference between the repolarization and activation time.  

Conduction velocity (CV) parallel (θ_L) and perpendicular (θ_T) to fiber direction was determined from the paced activation maps. Transmural CV (θ_TM, perpendicular to epicardium) was calculated from local activation times derived from electrograms recorded at the 3 non-stimulated electrode terminals of the separate needle electrode. Dispersion of conduction was assessed for LV and RV using the method described by Lammers et al.  The maximal dispersion in the septum (ΔtS_{max}) and the maximal LV transmural dispersion in ARI (ΔtM_{max}) were calculated. The activation patterns were determined by plotting the activation times (ΔV/Δt_{min}) referred to as the time difference to the first activated electrode.

An episode of Torsade de Pointes was defined as a sequence of at least five consecutive beats with a short coupling interval (250 ms) and a twisting QRS morphology in the surface ECG.

4. Hypertrophy and sodium current properties

Total heart to body weight (HW/BW) was determined in 12 CAVB and 9 SR dogs. Single myocytes were enzymatically isolated from the subepicardial layer of the left and right ventricle, as previously described. Following digestion, subepicardial cells were dissociated from a < 2mm slice dissected from the free wall, and used for cell size measurements. Of 4 control and 4 CAVB hearts cellular length and width of randomly selected isolated myocytes (≥ 50 cells per ventricle) were analyzed from light microscopic images at a resolution of 0.17 µm, using ImageJ 1.40 software.

Properties of the sodium current, I_{Na}, were measured in subepicardial cells using
standard whole-cell voltage-clamp technique. Patch pipettes were pulled from borosilicate glass and had a resistance of 1-3 MΩ when filled with internal solution. Currents were recorded with an Axopatch 200B amplifier, filtered at 5 kHz, and sampled and digitized at 10 kHz using a Digidata 1322A analog-to-digital converter. PClamp software (Axon Instruments) was used for voltage control, data acquisition and analysis. Capacitance and series resistance were partially compensated (60-80%). Current amplitude (pA) was normalized to cell capacitance (pF).

$I_{Na}$ was elicited from a holding potential of -120 mV at a frequency of 0.2 Hz. Steady-state inactivation curves were obtained by normalizing the peak current at each test potential to the maximal current. Activation curves were derived from current-voltage relations and normalized to maximal conductance. Values of activation and inactivation curves were fitted with a Boltzmann equation: $y=1\cdot(1+\exp[(V-V_{1/2})/k])^{-1}$ where $V_{1/2}$ is the potential of half maximal inactivation and $k$ is the slope factor. Data for recovery from inactivation were normalized and fitted using an exponential of the form: $y=y_0+\{1-\exp[-t\cdot\tau^{-1}]\}$ with $\tau$ being the time constant. Parameters of activation, inactivation and recovery were calculated and averaged from individual fitting of data.

For adequate voltage control, solutions with low concentrations of 5 mM Na$^+$ were used to reduce $I_{Na}$ amplitude. Pipettes were filled with (in mmol·L$^{-1}$): 5 NaCl, 120 CsCl, 20 TEA-Cl, 5 MgATP, 5 EGTA and 5 Heps; pH was adjusted to 7.2 with CsOH. The bath solution contained (in mmol·L$^{-1}$): 5 NaCl, 138 CsCl, 2.6 MgCl2, 1.8 CaCl2, 10 Heps, 10 glucose, and 20 μmol·L$^{-1}$ nifedipine, pH 7.4. Experiments were performed at 37°C.

5. Immunohistochemistry and histology

Biopsies from the LV and RV free wall were harvested from 5 CAVB and 5 SR dog hearts, submerged in Liquid nitrogen and stored at -80°C. Frozen biopsies were sectioned in 10 μm slices for immunolabeling and Picro Sirius Red staining. All slices were mounted on AAS (aminopropyltriethoxysilane)-coated glass slides.
Tissue sections were incubated with antibodies as reported previously \(^1\). After immunolabeling, sections were mounted in Vectashield (Vector Laboratories) and examined using a classic light microscope with epifluorescence equipment (Nikon Optiphot-2). The following antibodies were used; rabbit polyclonal anti-Cx43 (Zymed, Invitrogen, Breda, The Netherlands) and mouse monoclonal anti-dystrophin (Novocastra, Newcastle upon Tyne, UK). Secondary antibodies (Texas Red and FITC conjugated whole IgG) were purchased from Jackson Laboratories (Newmarket, UK).

For collagen staining, sections serial to the ones used for antibody-labeling were fixed with 4 % paraformaldehyde (in PBS, 30 minutes at RT) and stained with Picro Sirius Red as described previously. \(^2\)

5.1 Connexin43

Cx43 protein content was assessed in two ways. First, photomicrographs from LV and RV of CAVB (n=5) and control dogs (n=5) of Cx43 labeled sections (n=20 different samples at each site in the experimental groups) were loaded in ImageJ 1.40g (NIH, Bethesda, MD) and were subsequently transformed into RGB (i.e. Red Green Blue) stacks. In the green channel of the 256-leveled pictures a cut-off level of 60 was used to define Cx43 pixels. All pixels above the cut-off level were counted as a percentage of the total pixel count. \(^3\) This method allowed determination of Cx43 spatial heterogeneity by transforming photomicrographs into 8-bit gray pictures (256 gray levels) with a cut-off level of 60 to convert the gray scale into pure black (Cx43) and white (background) pictures. A custom script in Matlab (The MathWorks Inc, USA) was used to assess for each black pixel the shortest distance to the next black pixel in a circle around that pixel. The standard deviation of all shortest distances of all pixels was used as a measure of Cx43 spatial heterogeneity.

Cx43 quantification was also assessed in ventricular tissue lysates. Frozen tissue was pulverized in a custom made mortar cooled with liquid nitrogen and transferred to lysis buffer
(RIPA buffer with 150 mM NaCl, 10 mM Na₂HPO₄, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 1 mM EDTA, 50 mM NaF, 0.5% 1.5 mM aprotinin, and 0.5% 200 mM PMSF in isopropanol). Total protein content of the supernatant was analyzed with a BCA quantification essay. Total protein (25 µg per lane) was separated on a 10% SDS-PAGE gel and transferred onto nitrocellulose. Transfer was visualized and quantified using a reversible Ponceau-S staining, and signals were digitized using ScanWizard software. Membranes were blocked with 5% Protifar (Nutricia, Zoetermeer, the Netherlands) in 0.1% Tween-PBS and probed overnight at 4°C with a mouse monoclonal anti-Cx43 (BD Transduction Laboratories, San Jose, CA) or anti-N-cadherin antibody (Sigma, St. Louis, MO). Secondary antibody was HRP-conjugated anti-mouse antibodies (Bio-Rad, Hercules, CA). An enhanced chemiluminescence kit (ECL, Amersham, GE Healthcare, Little Chalfont, UK) was used for detection of the specific protein bands.

5.2 Quantification of collagen content

From Sirius Red stained sections digital photomicrographs were taken from up to 9 areas, depending upon the size of the section. Sirius Red staining was performed on 3 CAVB (sample size: 26 on LV and 20 on RV) and 4 control dog hearts (sample size: 36 on LV and 33 on RV). Quantification was done using ImageJ 1.40g software. Epicardial and perivascular fibrosis were excluded. For each section, Sirius Red staining in all photomicrographs was averaged.

6. Computer Simulations

We assessed conduction velocity (θₖ and θᵣ) in a linear strand of 100 longitudinally or transversally coupled subepicardial myocytes as described previously (Supplemental Figure 2). Briefly, individual cells of the strand were described with the Luo-Rudy dynamic model of mammalian subepicardial ventricular myocytes ('LRd model'). Entire cell length (for θₖ) or width
(for $\theta_T$) was used as the spatial discretization element in our computations, with elements connected by the lumped myoplasmic resistance (calculated from the myocyte dimensions and the myoplasmic resistivity of 150 Ω·cm) and gap junctional resistance (calculated from the myocyte dimensions and the gap junctional resistivity). Action potential propagation was elicited by injection of a 20% suprathreshold current pulse (2 ms duration) into the leftmost cell of the strand, at a frequency of 2 Hz. Simulations were run for a sufficiently long time to obtain steady-state. Conduction velocity was calculated from the difference in activation time of cells #20 and #80 of the strand.

Cell dimensions were based on experimentally obtained cell length for LV and RV control (i.e. SR) or CAVB myocytes, assuming that cell shape is that of a rectangular box. The number of membrane ionic channels was assumed to be proportional to membrane surface area (constant membrane ionic channel density). Based on our patch-clamp data, sodium channel density was set to 63.4% of LV control for LV CAVB myocytes, 77.2% of LV control for RV control myocytes, and 67.4% of LV control for RV CAVB myocytes. Gap junctional resistivity was set such that control values of 51.8, 45.2, 22.5 and 19.5 cm·s$^{-1}$ were obtained as observed experimentally under control conditions (cf. Table 2), and held constant in the remaining simulations.

7. Statistical analysis

Data were analyzed using SPSS software (SPSS 16, Chicago, USA). Statistical significance of differences was evaluated by unpaired Student’s t-test or ANOVA followed by post-hoc Bonferroni corrected t-test as appropriate. For data distributed non-normally the nonparametric Kruskal-Wallis test was used. Two-sided P-values <0.05 were considered statistically significant. All data are expressed as mean±SD.
REFERENCES


LEGENDS TO SUPPLEMENTAL FIGURES

Supplemental Figure 1 – Epicardial conduction velocities at different pacing cycle lengths

Epicardial CVs at different S\textsubscript{1}S\textsubscript{1} cycle lengths from 7 dog hearts (4 acute AV block (AAVB) and 3 CAVB). Longitudinal (θ\textsubscript{L}, squares) and transversal CV (θ\textsubscript{T}, dots) are depicted for RV (left panels) and LV (right panels) of AAVB (upper panels) and CAVB (lower panels). No significant differences in conduction velocity were found in AAVB or CAVB at LV and RV between pacing cycle lengths (2-way ANOVA). In both LV and RV of AAVB and CAVB, CVs were not dependent on pacing cycle length (S\textsubscript{1}S\textsubscript{1}: range 300-800 ms). This indicates that conduction velocity data recorded in SR and CAVB dogs can be compared.

Supplemental Figure 2 – Configuration of simulated strands

Strand configuration of longitudinally (A) or transversally (B) coupled ventricular myocytes used in the simulation study. Electrical characteristics of cells of the strand were described with the LRd model (see Extended Methods for further details). Myocyte length (L) and width (W) were based on experimentally obtained cell size data from isolated LV and RV myocytes of both control and CAVB dog hearts. Myoplasmic resistivity was set to 150 Ω·cm. R\textsubscript{j}, gap junctional resistivity.
Supplemental Table 1 – Electrophysiologic mapping data

<table>
<thead>
<tr>
<th></th>
<th>SR</th>
<th>CAVB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>LV</td>
</tr>
<tr>
<td>S1S2 pacing protocol</td>
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<tr>
<td>θL, cm·s⁻¹</td>
<td>50.8 ± 1.1</td>
<td>33.0 ± 1.5</td>
</tr>
<tr>
<td>θT, cm·s⁻¹</td>
<td>19.1 ± 1.6</td>
<td>15.1 ± 3.0</td>
</tr>
<tr>
<td>θTM, cm·s⁻¹</td>
<td>23.2 ± 5.7</td>
<td>26.2 ± 6.1</td>
</tr>
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</table>

Conduction parameters following the shortest premature stimulus (S2).
RV/LV, right and left ventricle, respectively; θL/θT/θTM, conduction velocity longitudinal or transversal to fiber direction and transmural conduction velocity, respectively. Repeated measurement analysis.
Supplemental Table 2 – *Electrophysiological parameters in different drug groups*

<table>
<thead>
<tr>
<th></th>
<th>Dofetilide</th>
<th>Almokalant</th>
<th>Ibutilide</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td></td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
<td></td>
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<tr>
<td>TdP incidence (%)</td>
<td>90</td>
<td>70</td>
<td>60</td>
<td>0.303</td>
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<tr>
<td>Average TdP duration (s)</td>
<td>5.5±1.2</td>
<td>8.8±11.2</td>
<td>10.6±8.6</td>
<td>0.438   **</td>
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<tr>
<td>Total TdP burden (s) after 10 min.</td>
<td>48.0±16</td>
<td>35.6±36</td>
<td>66.4±41</td>
<td>0.226   **</td>
</tr>
<tr>
<td>Number of required defibrillations (n)</td>
<td>2±7</td>
<td>1±6</td>
<td>1±2</td>
<td>0.331   ***</td>
</tr>
<tr>
<td>Δ APD¹⁰⁰% (increase after drug administration; ms)</td>
<td>98.9±44</td>
<td>120±46</td>
<td>130±60</td>
<td>0.385   **</td>
</tr>
</tbody>
</table>

*: χ² test  
**: ANOVA  
***: median±range; Kruskal-Wallis

Incidence of Torsade de Pointes arrhythmia in the chronic AV-block dog. Historical data from the CAVB model show comparable effects of the three class III drugs (i.e. ibutilide, dofetilide, and almokalant) in anesthetized dogs (same anesthetics). TdP induction was tested with the following dosages: dofetilide 0.025 mg/kg; almokalant 0.12 mg/kg; ibutilide 0.025 mg/kg. No statistically significant differences were found for the major electrophysiologic parameters.
Supplemental Figure 1: CV @ different CLs

RV

LV

AAVB

CAVB
Supplemental Figure 2: Simulated strands

A  Longitudinal
Stimulus

150 Ωcm
100 cells

B  Transversal
Stimulus

150 Ωcm
100 cells