Cellular mechanisms of acquired torsades de pointes in the hypertrophied canine heart : the substrate and the trigger

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Cellular Mechanisms of Acquired Torsades de Pointes in the Hypertrophied Canine Heart

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Cellular Mechanisms of Acquired Torsades de Pointes in the Hypertrophied Canine Heart The Substrate and the Trigger

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
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door

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*Dankzij mijn vader en moeder
Voor Ilya en ?*

Content

CHAPTER 1

- 9 Introduction

CHAPTER 2

- 31 **Repolarizing K^+ Currents I_{To1} and I_{Ks} Are Larger in Right than Left Canine Ventricular Midmyocardium** – Paul G.A. Volders, MD; Karin R. Sipido, MD, PhD; Edward Carmeliet, MD, PhD; Roel L.H.M.G. Späthjens, BS; Hein J.J. Wellens, MD, PhD; Marc A. Vos, PhD-Published in *Circulation*. 1999;99:206-210.

CHAPTER 3

- 41 **Cellular Basis of Biventricular Hypertrophy and Arrhythmogenesis in Dogs with Chronic Complete Atrioventricular Block and Acquired Torsade de Pointes** – Paul G.A. Volders, MD; Karin R. Sipido, MD, PhD; Marc A. Vos, PhD; Attila Kulcsár, PhD; S. Cora Verduyn, PhD; Hein J.J. Wellens, MD, PhD-Published in *Circulation*. 1998;98:1136-1147.

CHAPTER 4

- 65 **Downregulation of Delayed Rectifier K^+ Current in Dogs with Chronic Complete Atrioventricular Block and Acquired Torsades de Pointes** – Paul G.A. Volders, MD; Karin R. Sipido, MD, PhD; Marc A. Vos, PhD; Roel L.H.M.G. Späthjens, BS; Jet D.M. Leunissen-Beekman, BS; Edward Carmeliet, MD, PhD; Hein J.J. Wellens, MD, PhD-Accepted for *Circulation*. 1999.

CHAPTER 5

- 85 **Functional Upregulation of Na^+ - Ca^{2+} Exchange in Cardiac Hypertrophy May Contribute to Contractile Adaptation, But Also to Arrhythmogenesis** – Karin R. Sipido, MD, PhD; Paul G.A. Volders, MD; S.H. Marieke de Groot, MD, PhD; Mirella M.C. Molenschot, MS; Frans J. Van de Werf, MD, PhD; Hein J.J. Wellens, MD, PhD; Marc A. Vos, PhD. Manuscript submitted. 1999.

CHAPTER 6

- 109 **Similarities Between Early and Delayed Afterdepolarizations Induced by Isoproterenol in Canine Ventricular Myocytes** – Paul G.A. Volders, MD; Attila Kulcsár, PhD; Marc A. Vos, PhD; Karin R. Sipido, MD, PhD; Hein J.J. Wellens, MD, PhD; Ralph Lazzara, MD; Bela Szabo, MD, PhD-Published in *Cardiovasc Res*. 1997;34:348-359.

CHAPTER 7

- 133 General Discussion Part I: Altered Electrical Properties in the Dog with Chronic Complete Atrioventricular Block: The Substrate and the Trigger for Torsades de Pointes

CHAPTER 8

- 145 General Discussion Part II: Progress in the Understanding of Cardiac Afterdepolarizations and Torsades de Pointes: Time to Revise Current Concepts

- 185 Summary

- 189 Samenvatting

- 193 Dankwoord

- 197 Curriculum Vitae

- 199 Publications

Introduction

Content

- 1.1 Sudden Cardiac Death and the Long-QT Syndromes
- 1.2 Historical Remarks on Torsades de Pointes: Congenital Versus Acquired Long-QT Syndromes
- 1.3 Cellular Basis of Ventricular Repolarization
- 1.4 Aims of This Thesis: The Substrate and the Trigger
- 1.5 The Dog with Chronic Complete Atrioventricular Block
- 1.6 Methods
- 1.7 References

1.1 Sudden Cardiac Death and the Long-QT Syndromes

Despite significant breakthroughs in cardiovascular medicine in the last decades, diseases of the heart and vessels, including stroke, continue to claim a high morbidity and death toll in the Western world. In the United States they account for 42% of all deaths (455,000 men and 505,000 women in 1995), exceeding by far the mortality from any other disease.^{1,2} Although the incidence of coronary artery disease as a primary cause of mortality has gradually declined since the 1960s, a comparison of mortality data from 1973 and 1993 reveals that this decline is most apparent in the young and middle aged. Significant improvements in acute treatment and secondary prevention programs are the main reasons that these patients live longer after a heart attack or stroke, or after being diagnosed with hypertension, but this has only postponed the cardiovascular mortality with a few years.² The resultant growing number of patients with congestive heart failure puts a burden on health-care facilities in 1999 and has far-reaching consequences for the next century.

Often the first manifestation of cardiovascular disease is sudden death. Sudden cardiac death is responsible for 300,000 to 400,000 victims per year in the United States.³ In the Maastricht area, with approximately 182,000 inhabitants, the overall yearly incidence of out-of-hospital sudden cardiac arrest (death rate 93%) in the age of 20 to 75 years is 1 per 1000 people.⁴ Over 90% of the fatalities occur in patients with known or previously unrecognized coronary artery disease or cardiomyopathies. In the remaining 5 to 10% no evidence of structural heart disease can be found.⁵

Cardiac arrhythmias are a frequent cause of sudden death. In a study of patients who had died suddenly and in which the cause of death was arrhythmic (as evident from Holter-recordings), ventricular tachyarrhythmias were the terminal event in 83% of the cases, whereas bradyarrhythmias occurred in 17%.⁶ Three forms of tachyarrhythmias were distinguished: (1) primary ventricular fibrillation (10% of the 83%); (2) ventricular tachycardia or flutter precipitating ventricular fibrillation (75%); and (3) torsades de pointes (15%).⁶ Fortunately, most often, ventricular arrhythmias have less detrimental consequences. Depending on the type and nature of these rhythm perturbations, some patients have no symptoms at all, whereas others experience palpitations, dizziness, lightheadedness, presyncope or syncope.

This thesis deals with the basic electrophysiological mechanisms of a life-threatening ventricular arrhythmia called '*torsades de pointes*' (Figure 1). On the electrocardiogram, torsades de pointes appears as a polymorphic tachycardia characterized by a distinctive pattern of undulating peaks of sequential QRS complexes and T waves (by definition ≥ 5 beats) that twist around the baseline.⁷ The arrhythmia varies in duration, lasting several seconds to minutes. It can stop spontaneously, change into a more monomorphic tachycardia, or degenerate into ventricular fibrillation thus leading to sudden death. Patients suffering and surviving from torsades de pointes describe sensations that range from mild discomfort to loss of consciousness. Torsades de pointes typically occurs in hearts characterized electrocardiographically by excessive QT prolongation and

Figure 1. – Representative example of acquired torsades de pointes in a 66-year old female patient, who was given ibutilide intravenously to terminate an episode of atrial fibrillation. As shown, the arrhythmia developed in the setting of sinus rhythm and a prolonged QT interval, and is preceded by a short-long-short RR interval. Courtesy of Dirk W. Donker, MD (Department of Cardiology, Academic Hospital Maastricht).

T-wave abnormalities, in the absence of changes of the QRS complex. Collectively, these states are called the ‘*long-QT syndromes*’. The long-QT syndromes are congenital or acquired in nature.

The *congenital* long-QT syndromes are a unique group of functional cardiac repolarization disorders with the following common characteristics: (1) abnormal prolongation of the electrocardiographic QT interval due to the inherited malfunctioning of ion channels in the cardiac myocytes; *plus* (2) increased susceptibility to torsades de pointes.

The *acquired* long-QT syndromes can be broadly defined as a heterogeneous group of cardiac repolarization disorders with the following common characteristics: (1) the development of abnormal prolongation of the electrocardiographic QT interval after exposure to pathophysiological and/or pharmacological influences in previously normal myocardium; *plus* (2) increased susceptibility to polymorphic ventricular arrhythmias, notably torsades de pointes. A typical example of acquired torsades de pointes is shown in Figure 1.

The incidence of the congenital long-QT syndrome in the general population is low. In the International Long-QT Syndrome Registry,⁸⁻¹⁰ the number of enrolled

symptomatic and asymptomatic family members with a confirmed diagnosis is approaching 10,000 worldwide. Most probably, the true incidence is much higher in various regions than suggested by this figure. Cases have been diagnosed in every continent and race. The overall male-to-female ratio of involvement is 31 : 69, indicating a clear female predominance.¹¹ The incidence of the acquired long-QT syndromes is much harder to estimate due to the multiple etiology. Undoubtedly, acquired forms of the long-QT syndrome are much more common than the congenital type. In the Department of Cardiology at the Academic Hospital Maastricht with 3300 clinical admissions per year, the number of admissions due to acquired torsades de pointes is estimated at approximately 10 per year (Dr. A.P.M. Gorgels, personal communication).

Several aspects make the long-QT syndrome a very interesting disease. First of all, the occurrence of syncope and sudden death are dramatic manifestations having a major impact on patient and families. Secondly, the syndrome is a Rosetta stone for the mechanisms by which modulation of the autonomic nervous system enhances or prevents ventricular tachyarrhythmias and sudden cardiac death.¹² Thirdly, it is the prime example of how molecular biology, and ion-channel, cellular, and organ physiology can cooperate in unraveling a serious clinical problem.

1.2 Historical Remarks on Torsades de Pointes: Congenital Versus Acquired Long-QT Syndromes

Congenital Long-QT Syndromes

Probably the first report on torsades de pointes in the medical literature stems from 1856. Meissner¹³ described a congenitally deaf girl who collapsed and died while being rebuked in public. Two siblings of the girl had previously died suddenly during emotional stress. More than 100 years later congenital deaf-mutism combined with syncope or sudden death during exercise or emotion was linked to functional heart disease characterized by pronounced prolongation of the QT interval.¹⁴ In a landmark article, Drs. Jervell and Lange-Nielsen¹⁴ stated that *'The unusual clinical symptoms, the exceptional electrocardiographic findings, and the serious outcome of the illness, together, represent a characteristic syndrome ...'* later to become known as the Jervell-Lange-Nielsen long-QT syndrome. In 1963 and 1964, Romano et al^{15,16} and Ward¹⁷ reported independently that inherited QT prolongation and ventricular arrhythmias can also exist without deafness. It is now known that this type is the most frequently encountered congenital long-QT syndrome with an autosomal dominant mode of inheritance, whereas the Jervell-Lange-Nielsen type inherits by autosomal recessive mode.^{10,18} In 1970, Garza et al¹⁹ described another case of the Romano-Ward syndrome. A comatose 3-year-old boy was admitted to the hospital with *'a syndrome of prolonged Q-T interval, potentially fatal episodes of ventricular arrhythmias, and normal hearing'*. A brother and the mother of this patient had previously experienced multiple syncopes themselves during moments of vigorous exercise. The authors endeavored to

perform physiological investigations on the irreversibly brain-damaged boy in their search for an arrhythmia prophylaxis for the brother and mother. The responses to epinephrine, phenylephrine, isoproterenol and propranolol indicated a strong adrenergic dependence of the torsades de pointes in the young patient and led to a tailored, successful treatment of his relatives with propranolol.¹⁹ This concept was advanced for its time and was partly derived from the experimental studies by Yanowitz and associates,²⁰ who had measured changes of the electrocardiogram and the ventricular refractory period following alterations of the sympathetic tone in dogs. Right-stellate ganglionectomy or left-stellate stimulation produced QT prolongation and increased T-wave amplitudes, whereas right-stellate stimulation or left-stellate ganglionectomy accentuated T-wave negativity without measurable changes of the QT interval.²⁰ Soon thereafter, a case report in the *New England Journal of Medicine* proved the clinical applicability of left-stellate ganglionectomy for the antiarrhythmic treatment of the congenital long-QT syndrome and it was hypothesized that an '*endogenous sympathetic-nerve imbalance*' could explain part of the etiology ('*sympathetic-imbalance hypothesis*').²¹ Wellens et al²² deserve credit for proposing an additional, more intracardiac, mechanism of proarrhythmia. In 1972, they described a patient with syncopal attacks due to torsades de pointes on being awakened from sleep by auditory stimuli (thunderclap, alarm clock, falling bedpan, rock-and-roll music). In this famous case of, what was most likely, the Romano-Ward syndrome, the authors investigated whether increased asynchrony in refractoriness of different areas of the ventricles could predispose to the arrhythmic activity. Although no direct evidence for this could be found during electrical stimulation of the heart, the authors realized that '*... this does not exclude the possibility that ... asynchrony in refractoriness of ventricular muscle was present between subendocardial and subepicardial layers, nor that this occurred following neural stimulation or during the first beats following sudden changes in heart rate*'.²² Wellens et al also showed that the arrhythmia could be completely prevented by the β -blocking drug propranolol.²²

Around the same time another cause for arrhythmias was reported when under experimental conditions afterdepolarizations were found to be responsible for abnormal impulse generation in myocardial tissue preparations.^{23,24} A putative proarrhythmic role for afterdepolarizations in the congenital long-QT syndrome was investigated by Schechter et al.²⁵ In a 23-year-old female patient, they recorded electrograms from the ventricular endocardium during emotional upset and epinephrine infusion. At various sites in the left and right ventricle, prominent diastolic humps on the electrogram, which occurred after the electrocardiographic T wave, were observed at baseline and became more accentuated during adrenergic stimulation. Termination of the epinephrine infusion together with the administration of diazepam attenuated the potentials. Based on these clinical observations and extrapolation from cellular electrophysiological events, adrenergic-induced afterdepolarizations were taken to be involved, at least partly, in the generation of ventricular ectopic beats and the initiation of torsades de pointes.²⁵ Many subsequent studies with monophasic-action-potential recordings confirmed this hypothesis (eg, References 26 through 30). It was in 1975

when Schwartz and Malliani³¹ identified alternans of the T wave as a second electrocardiographic characteristic of the long-QT syndrome. They made this observation in a 9-year-old symptomatic girl and were able to reproduce it in anesthetized cats during unilateral left-stellate-ganglion stimulation.³¹ In these years, the call for an international registry for the congenital long-QT syndrome intensified and in 1979 Drs. Moss, Crampton and Schwartz launched a prospective study to define (1) the natural history of the disease in asymptomatic patients with prolonged QT intervals not receiving therapy, (2) the long-term efficacy of therapy in symptomatic patients, and (3) epidemiological and genetic characteristics.⁸ The registry, expected to last 25 years, enrolled more than 728 families thus far¹⁰ and served as the database for many important publications. A major breakthrough came in 1991, when Keating et al³² demonstrated linkage of the long-QT syndrome to the Harvey *ras-1* gene on the short arm of chromosome 11 in a large family. In 1994, Towbin et al³³ confirmed this for 15 of 23 families, whereas in 4 families linkage to the Harvey *ras-1* gene was definitively excluded. Thus, conclusive evidence for genetic heterogeneity in the long-QT syndrome was found. These studies provided a second hypothesis on the pathogenesis of the syndrome: '*the intracardiac-abnormality hypothesis*', in which defective ionic currents in ventricular cells are the cause of a low repolarization reserve leading to QT prolongation and T-wave alternans. Since the start of this PhD project in 1993, 5 loci have been associated with the Romano-Ward syndrome through genetic studies worldwide and they are located on the chromosomes 3, 4, 7, 11, and 21.¹⁸ Four disease genes, each encoding an ion-channel protein, could be identified: *KvLQT1* encoding the α subunit of the I_{Ks} -channel protein (chromosome 11; LQT1)³⁴; *HERG* encoding the I_{Kr} -channel protein (chromosome 7; LQT 2)³⁵; *SCN5A* encoding the cardiac Na^+ channel (chromosome 3; LQT3)³⁶; and *KCNE1* encoding *minK*, the ancillary subunit of the I_{Ks} -channel complex (chromosome 21; LQT5)³⁷. The gene at the chromosome-4 locus (LQT4) has not yet been identified. The reader is referred to Figures 2 and 3 for an outline of the functional relevance of these ionic currents for the ventricular action potential. Determination of the genotypes of 541 members of 38 families of the International Long-QT Syndrome Registry revealed that 112 had mutations at the LQT1 locus, 72 had mutations at the LQT2 locus and 62 had mutations at the LQT3 locus,¹⁰ making LQT1 the most frequently encountered form. The other 295 members were either not affected or their genotype could not be clarified. Reports on new mutations at these loci appear in the literature almost every month. A final aspect is that ventricular wall motion abnormalities have been observed in patients with the congenital long-QT syndrome during echocardiography and these can be abolished with Ca^{2+} -channel blockers.³⁸

Figure 2. – Schematic diagram of the Luo-Rudy guinea-pig ventricular cell model,⁷⁷ showing the sarcolemmal ionic currents and intracellular ionic fluxes that are relevant for the action potential.

Sarcolemmal currents: I_{Na} indicates fast Na^+ current; $I_{Ca(L)}$, L-type Ca^{2+} current; $I_{Ca(T)}$, T-type Ca^{2+} current; I_{Kr} , rapidly activating component of the delayed rectifier K^+ current; I_{Ks} , slowly activating component of the delayed rectifier K^+ current; I_{K1} , inward rectifier K^+ current; I_{Kp} , 'plateau K^+ current'; I_{NaK} , Na^+ - K^+ pump current; I_{NaCa} , Na^+ - Ca^{2+} exchange current; $I_{p(Ca)}$, Ca^{2+} ATPase in the sarcolemma (currently it is unclear whether this is an electrogenic current); $I_{Na,b}$, 'background Na^+ current'; $I_{Ca,b}$, 'background Ca^{2+} current'; and $I_{ns(Ca)}$, Ca^{2+} -activated nonspecific cation current. Intracellular fluxes or delays (these are not electrogenic currents): I_{up} indicates Ca^{2+} ATPase for uptake of Ca^{2+} into the network sarcoplasmic reticulum (NSR); I_{rel} , Ca^{2+} release from the junctional sarcoplasmic reticulum (JSR) to cytoplasm; I_{leak} , Ca^{2+} leakage from JSR; and I_{tr} , Ca^{2+} translocation from NSR to JSR. Calmodulin, troponin and calsequestrin are intracellular Ca^{2+} buffers. Not on this diagram, but important for the canine ventricular action potential are I_{To} , transient outward K^+ current; $I_{Ca(Cl)}$, Ca^{2+} -activated Cl^- current; $I_{K(ACh)}$, acetylcholine-sensitive K^+ current. Courtesy of Yoram Rudy, PhD (Department of Biomedical Engineering, Case Western Reserve University, Cleveland, Ohio, USA). Reproduced with permission of the American Heart Association®.

Acquired Long-QT Syndromes

Initial studies on acquired forms of the long-QT syndrome appeared in the literature in the 1920s and 1930s.³⁹⁻⁴² It is remarkable to find, and particularly interesting for the scope of this thesis, that in almost every case these were reports on patients with atrioventricular block ('*auriculoventricular dissociation*'). An active person in this field was Dr. Sidney P. Schwartz from the Montefiore Hospital in New York City, who elaborated on the clinical and electrocardiographic characteristics of what was called '*transient ventricular fibrillation*' at that time. In 1934, Schwartz and Jezer⁴³ criticized other specialists for their supposed therapeutical administration of quinine and quinidine to patients with atrioventricular block and transient ventricular fibrillation, because this was '*based on very meager clinical experience*' and would be rather pro- than

Figure 3. – Schematic representation of inward and outward ionic currents involved in the canine ventricular action potential, resting membrane potential and cytoplasmic Ca^{2+} transient under control conditions.

The numbers 0 through 4 indicate the different phases of the spike-and-dome action potential: 0, upstroke; 1, fast early repolarization phase with notch; 2, plateau phase; 3, repolarization phase; 4, resting membrane potential. The relative contribution of each of the currents varies depending on the physiological conditions. The approximate time courses are shown symbolically. The current amplitudes are not to scale. For legends to the currents' denotation see Figure 3. Vertical scale bar left to action potential indicates 0 to -50 mV. Vertical thin dashed lines through whole figure refer to 4 time points in the action potential. Adapted from references 55, 56, and 78 through 92.

antiarrhythmic. To make their point, the authors injected quinine and quinidine into two patients and, unfortunately, both collapsed from torsades-like arrhythmias. It was judged that *'the use of such drugs by this method is contraindicated in such patients'*. In a later paper, Schwartz⁴² described 6 patients with recurrent syncopal attacks due to torsades-like tachycardias, however now in the absence of drugs. These patients were admitted with second or third degree atrioventricular block of unrevealed cause, and had very low ventricular rates (*'the average basic ventricular rate was 25 beats per minute without the interposition of premature ventricular beats'*), and long QT intervals. The term *'prefibrillatory mechanism'* was used to denominate the alterations in heart rhythm that preceded the arrhythmias in all 6 patients. *'When the heart rate has thus been accelerated gradually to a certain level of speed, it is then suddenly interrupted by at first single and then recurrent groups of ventricular oscillations of wide aberrancy that herald the approach of a period of ventricular fibrillation'*.⁴² It was learned that *'extrinsic nerves'* had a major influence on the arrhythmia initiation and in 1950 Schwartz and de Sola Pool⁴⁴ published on the effects of bodily rest, atropine and exercise on transient ventricular fibrillation. *'One patient ... had experienced several seizures of transient ventricular fibrillation following the type of exertion associated with "straining at stool" or when walking rapidly up an incline'*. This was in line with a previous report by Dock,⁴⁰ who observed such seizures in a patient after injection of epinephrine. In 1966 the term *'transient ventricular fibrillation'* was definitively replaced by *'torsades de pointes'*. Dr. Dessertenne⁷ of the Hôpital Lariboisière in Paris coined this expression after observing the *'électrocardiogramme en crise'* of an 80-year-old lady with heart failure, complete atrioventricular block and frequent syncopal attacks. Interestingly, when the patient was administered potassium chloride and put at rest, she was symptomless for three days. In the view of Dessertenne, torsades de pointes was the twisting of two opposing foci of electrical activity in the ventricles and was distinguishable from ventricular fibrillation, although the torsades could end so.⁷ In the years that followed, and until this moment, a vast number of case reports and clinical studies on acquired torsades de pointes highlighted the notion that not only the condition of chronic atrioventricular block, but also various other pathophysiological and pharmacological influences, including antiarrhythmic and non-cardiovascular drugs, predispose to the arrhythmia. For excellent comprehensive reviews we refer to Jackman et al.^{26,45,46} The awareness of a poor basic electrophysiological understanding of torsades de pointes prompted the development of surrogate models of the long-QT syndromes. In 1983, Brachmann et al.⁴⁷ proposed the use of intravenous cesium chloride for the in-vivo and in-vitro study of *'bradycardia-dependent multiform ventricular tachycardia'* in anesthetized dogs. These investigators invoked an important role for prolonged repolarization and afterdepolarization-dependent triggered activity in the initiation and maintenance of the tachycardia. Subsequently, in various other models, torsades de pointes was induced by class-Ia or class-III antiarrhythmic agents, or other substances with related actions.⁴⁸ A great advantage of these animal models was the opportunity to study the cellular and ionic basis of in-vivo arrhythmias in isolated cardiac tissues and myocytes.

One additional matter deserves mentioning. Ventricular arrhythmias and sudden cardiac death are major concerns in the clinical management of cardiac hypertrophy and failure.^{49,50} In contemporary discussions on the mechanisms of arrhythmias in these conditions, an important role is attributed to action-potential prolongation and increased regional dispersion of repolarization.⁵¹ The findings of QT-interval and repolarization labilities in patients with ischemic and nonischemic dilated cardiomyopathy,^{52,53} and other cardiac disorders with hypertrophy or failure, raises the question whether also here the substrate for arrhythmias is related, at least partly, to a lower repolarization reserve in the myocardial cells. Alterations in K^+ currents have indeed been observed in such patients,⁵⁴ and similar ionic changes occur in many experimental animal models with hypertrophy or failure of the heart.⁵⁵ Although the long-QT syndromes and the cardiomyopathies of overload are clearly distinctive clinical entities, mechanistic understanding of their common endpoints of excessive QT prolongation and T-wave abnormalities warrants similar basic electrophysiological studies.

1.3 Cellular Basis of Ventricular Repolarization

The ionic mechanisms responsible for the repolarization of single ventricular cells are illustrated in Figures 2 and 3. In the spike-and-dome action potential of the epi- and midmyocardium of the dog, the experimental animal of this thesis, a large transient outward current has been held responsible for the phase-1 repolarization (causing the typical notch; see Figure 3). This current is composed of a 4-aminopyridine-sensitive component (I_{TO} , also designated I_{TO1}) and a Ca^{2+} -activated Cl^- current ($I_{Ca(Cl)}$). During the plateau phase (phase 2), there is a delicate balance of inward and outward currents. Inward currents can be carried through the Na^+ channel, the Ca^{2+} channels, and the Na^+-Ca^{2+} exchanger. Outward currents during the plateau are carried by a number of K^+ channels, a Cl^- channel, and the Na^+-K^+ pump. For the initiation of phase 3 of the action potential, the contributions of rapidly and slowly activating delayed rectifier K^+ currents are of key importance. Furthermore, the Na^+-K^+ -pump current and the inward-rectifier K^+ current are important participants. An acetylcholine-sensitive K^+ current is also active.⁵⁶ The stable resting membrane potential is maintained by the inward-rectifier K^+ current, Na^+-K^+ -pump current, acetylcholine-sensitive K^+ current and possibly also Na^+-Ca^{2+} -exchange current (depending on the intracellular Na^+ and Ca^{2+} concentrations).

Electrophysiological studies on sliced myocardial-tissue preparations and arterially-perfused wedges indicate that voltage gradients in the ventricles, generated by the joint action of the myocytes, play an important role in the genesis of the electrocardiographic T wave.⁵⁷ Regional differences in baseline electrical properties and in responses to pharmacological and pathophysiological influences characterize the ventricular myocardium. Such differences are found in the transmural and apico-basal ventricular myocardium, in Purkinje fiber-versus-working myocyte comparisons, in

the transseptal myocardium, and in left-versus-right ventricular comparisons. The transmural myocardium is known for its prominent disparity of action-potential configuration and duration between epicardial, midmyocardial and endocardial layers. These three layers differ principally with respect to phase-1 and phase-3 repolarization characteristics. Whereas a prominent notch shapes the typical spike-and-dome action potential of the epicardium and midmyocardium, this feature is absent in the endocardium. Another electrophysiological distinction among the three layers is their action-potential duration (APD).^{58,59} In isolated tissue and single-cell studies, action potentials appeared to be longest in the midmyocardium, and APD prolongation in response to slow pacing rates, class Ia⁶⁰ and class III agents⁶¹ was more pronounced in this layer than in either the epi- or endocardium. The longer APD of midmyocardial cells, versus epi- and endocardial myocytes, has been explained on the basis of a lesser contribution of the slowly activating component of the delayed rectifier K^+ current⁶¹ and possibly also because of a larger background Na^+ current in the midmyocardial cells. The rapidly activating component of the delayed rectifier K^+ current and the inward rectifier K^+ current are not different in the three layers. As mentioned earlier (section 1.2), the attenuation of either component of the delayed rectifier K^+ current by pharmacological inhibition or congenital or acquired downregulation has major action-potential-prolonging consequences and can be associated with the generation of early afterdepolarizations or plateau arrests.

In recent investigations, Yan and Antzelevitch⁶² used an experimental setup in which transmembrane action potentials recorded from epi-, mid- and endocardial sites, as well as from Purkinje fibers could be temporally correlated with a transmural 'electrocardiogram'. Under baseline conditions the early repolarization of the epicardial action potential coincided with the peak of the T wave, whereas the late repolarization of the midmyocardial action potential was temporally aligned with the ending of the T wave. In acquired QT prolongation by hypokalemia, *dl*-sotalol or both, these gradients were accentuated due to the more pronounced response of the midmyocardium and they caused a marked increase of the peak-to-end interval of the T wave. Often the T wave showed a widened and bifurcated aspect, suggesting the presence of a pathophysiological U wave, but this was more likely the second component of an interrupted T wave.⁶² Patterns of prolonged and bifurcated T waves have long been known for clinical acquired and congenital long-QT syndromes.^{63,64}

1.4 Aims of This Thesis: The Substrate and the Trigger

Previous in-vivo investigations on acquired torsades de pointes by our group indicated that electrical remodeling in the ventricular myocardium of dogs with chronic complete atrioventricular block causes an enhanced susceptibility to the arrhythmia ('*The Substrate*'). The propensity to abnormal impulse formation in this substrate is likewise enhanced and is related to early and delayed afterdepolarizations ('*The Trig-*

ger'). It was the aim of this thesis to investigate the cellular basis of *the substrate* and *the trigger* in dogs with chronic complete atrioventricular block and acquired torsades de pointes.

1.5 The Dog with Chronic Complete Atrioventricular Block

The dog with complete atrioventricular block has been used by various members of the Department of Cardiology in Maastricht since the beginning of the 1980s and is very suitable for the investigation of ventricular arrhythmias. Early studies were directed to the understanding of ventricular impulse formation during digitalis intoxication.⁶⁵ Subsequently, the role of programmed electrical stimulation and antiarrhythmic drugs for the identification and treatment of delayed-afterdepolarization-dependent ventricular arrhythmias were highlighted.⁶⁶ The administration of class-III antiarrhythmic agents, alone or in combination with programmed electrical stimulation, appeared a very suitable means to induce acquired torsades de pointes in a reproducible manner in this model.⁶⁷ The relative importance of action-potential prolongation, increased dispersion of repolarization, early afterdepolarizations and multiple ventricular ectopic beats for the initiation and perpetuation of torsades de pointes were appreciated.⁶⁸ Recently, it became clear that electrophysiological and functional adaptations in the chronic, hypertrophic phase of complete atrioventricular block predisposed to triggered activity, and that electromechanical and mechano-electrical interactions were of prime importance for the arrhythmogenesis under dynamic conditions.⁶⁹ The results of these lines of investigation were published in a number of articles (eg, References 67, 70 and 71) and led to the hypothesis that the process of electrical remodeling, causing increased arrhythmogenesis in a few weeks time, is determined by changes in ion-channel function. This was the start of the present work.

1.6 Methods

Isolation of Single Canine Ventricular Myocytes

The cell preparations used for this thesis are based on the isolation procedure originally described for rat heart by Powell and Twist in 1976,⁷² and modified by the same⁷³ and other⁷⁴⁻⁷⁶ investigators afterwards.

In our laboratory, the hearts of anesthetized dogs are quickly excised and washed with cold, cardioplegic solution. One or two coronary arteries are rapidly cannulated to perfuse the tissue with cardioplegic solution, nominally Ca^{2+} -free standard-buffer solution and collagenase solution at low $[\text{Ca}^{2+}]$ (50 $\mu\text{mol/L}$). These steps take approximately 40 minutes. After washout with enzyme-free low- $[\text{Ca}^{2+}]$ solution, the tissue is perfused with standard-buffer solution at 1.8 mmol/L $[\text{Ca}^{2+}]$. Finally, the tissue is minced into suspension, filtered and washed several times. Ca^{2+} -tolerant and electro-

Figure 4. – Photomicrograph of enzymatically isolated myocytes from the left ventricle of a normal adult dog.

These are typical examples of the cells used for electrophysiological studies. They have clear rod-shaped contours, transparent cytoplasms, and marked striations. The myocyte in the center has a size of $170 \times 25 \mu\text{m}$ (length $\times$ width). Magnification $\times 1000$. Courtesy of Jack P.M. Cleutjens, PhD and Mat J.A.P. Daemen, MD, PhD (Department of Pathology, Academic Hospital Maastricht).

Figure 5. – Laboratory of cellular cardiac research in Maastricht.

The left panel is a schematic drawing of the assembly of the various components of the upper photographs. A indicates the inverted fluorescence microscope; B, the microscope table with perfusion chamber for the myocytes; C, perfusion system with water-jacketed heat exchanger for inflow of superfusate to the chamber (B); D, fiber-optic light source; E, micromanipulators for coarse and fine positioning of the microelectrodes; F, video camera; G, headstage (preamplifier) with holder and microelectrode; H, vibration-isolated work station; I, stimulator; J, microelectrode amplifiers; K, video edge-motion detector; L, oscilloscope; M, analog-to-digital converter; N, computer with pCLAMP6 software (Axon Instruments, Inc) for automated waveform control and data acquisition; O, photomultipliers; P, box with Xenon-arc lamp and monochromators; Q, computer with software for automated fluorescence wave-length control of monochromators.

Figure 6. – Photomicrograph of glass microelectrodes.

The 'blunt' patch electrode (left ; borosilicate glass; tip diameter 1-2 μm) is used for whole-cell voltage clamp and typically has a resistance of 1.0 to 3.0 $\text{M}\Omega$ when filled with internal solution. The 'sharp' microelectrode (right; standard glass; diameter <1 μm) is used for membrane-potential recordings and typically has a resistance of 30 to 60 $\text{M}\Omega$ when filled with 3.0 mol/L KCl.

physiologically useful single cells are obtained in a reproducible manner by this procedure (see Figure 4). For more detailed descriptions of the cell-isolation procedures, the reader is referred to the following chapters.

Laboratory of Cellular Cardiac Research

For the purpose of cellular electrophysiological, contraction and ion measurements a new experimental setup was built in 1995 (Figure 5). The setup is constructed around an inverted microscope and allows for the recording and analysis of action potentials, ionic currents (whole-cell voltage clamping) and cell contraction (video edge-motion detection) in single cardiac myocytes. The addition of an epifluorescence system has expanded the arsenal to detect and quantify intracellular ions (Ca^{2+} , Na^+ , K^+ , H^+) at high-time resolution. Currently, two different types of experiments can be conducted: (1) studies with (high-resistance, 3 mol/L KCl) sharp microelectrodes (Figure 6) to record action potentials and contractions, in which the intracellular milieu is minimally altered, and (2) studies with patch electrodes (Figure 6) for the recording of

ionic currents and $[Ca^{2+}]_i$ (or other ions), in which the intracellular milieu is purposefully manipulated. A detailed description of the actual experimental setup of the various studies is given in the different chapters.

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Repolarizing K^+ Currents I_{TO1} and I_{Ks} Are Larger in Right Than Left Canine Ventricular Midmyocardium

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Abstract

Background: The ventricular action potential exhibits regional heterogeneity in configuration and duration (APD). Across the left ventricular (LV) free wall this is explained by differences in repolarizing K⁺ currents. However, the ionic basis of electrical nonuniformity in the right (RV) versus LV is poorly investigated. We examined transient outward (I_{TO1}), delayed (I_{Ks} and I_{Kr}), and inward rectifier K⁺ currents (I_{K1}) in relation to action-potential characteristics of RV and LV midmyocardial (M) cells of the same adult canine hearts.

Methods and Results: Single RV and LV M cells were used for microelectrode recordings and whole-cell voltage clamping. Action potentials showed deeper notches, shorter durations at 50% and 95% of repolarization, and less prolongation upon slowing of the pacing rate in RV than LV. I_{TO1} density was significantly larger in RV than LV, while steady-state inactivation and rate of recovery were similar. I_{Ks} -tail currents, measured at -25 mV and insensitive to almokalant (2 μ mol/L), were considerably larger in RV than LV. I_{Kr} , measured as almokalant-sensitive tail currents at -50 mV, and I_{K1} were not different in the two ventricles.

Conclusions: Differences in K⁺ currents may well explain the interventricular heterogeneity of action potentials in M layers of the canine heart. These results contribute to a further phenotyping of the ventricular action potential under physiological conditions.

Introduction

Regional heterogeneity of the action-potential configuration and duration (APD) characterizes the ventricular myocardium in large mammals, including humans.¹⁻³ A prominent notch shapes the typical spike-and-dome action potential of the epicardium and midmyocardium (M layer), but is absent in the endocardium. A relatively large transient outward current, containing a 4-aminopyridine-sensitive component (I_{TO1}) and Ca^{2+} -activated Cl^- current, is mainly responsible for this notch. Another in-vitro electrophysiological distinction is the longer APD of midmyocardium and its pronounced increase in response to slow pacing rates, class Ia and class III agents.^{1,4,5} These repolarization characteristics have been explained on the basis of a lesser contribution of the slowly activating component (I_{Ks}) of the delayed rectifier K^+ current in M cells,^{4,6} while the rapidly activating component (I_{Kr}) and the inward rectifier current (I_{K1}) appear similar in the three transmural layers.⁷

Only limited information is available on action-potential and ionic differences in right (RV)-versus-left ventricular (LV) comparisons. A larger I_{TO1} in RV versus LV epicardial cells has been correlated with a larger notch in the former cell type.⁸ Since an interventricular comparison of K^+ currents in M cells is lacking, we examined action potentials and the K^+ currents I_{TO1} , I_{Ks} , I_{Kr} , and I_{K1} in RV and LV M cells of the same adult canine hearts.

Methods

Sixteen mongrel dogs of either sex (26 ± 1 kg) were anesthetized and received perioperative care as described.⁹ Thoracotomy was performed and hearts (225 ± 12 g) were quickly excised. RV and LV M cells were obtained by simultaneously cannulating the left-anterior-descending and right coronary arteries.¹⁰ After ≈ 30 minutes of collagenase perfusion, the epicardial surface layer was removed from both wedges until a depth of ≥ 3 mm,^{4,7} and softened tissue samples were pipetted from the M layer underneath, while contamination with the endocardium was avoided. Samples were gently agitated, filtered and washed. Isolated myocytes were stored at room temperature in standard-buffer solution.

The setup was built around an inverted microscope.¹⁰ Microelectrodes (standard glass) had resistances of 30 to 60 $M\Omega$ when filled with 3.0 mol/L KCl. Intracellular pacing was done at various cycle lengths (CLs). For the recording of ionic currents, we used the whole-cell variant of the patch-clamp technique. Patch pipettes (borosilicate glass) had resistances of 1.0 to 3.0 $M\Omega$ when filled with internal solution. Experiments were performed at 37°C. Cell capacitance, measured by hyperpolarizing steps from -60 mV, was similar in RV ($n=27$) and LV M cells ($n=25$) being 226 ± 12 and 226 ± 11 pF, respectively ($P=NS$). L-type Ca^{2+} current was blocked with nifedipine (5 μ mol/L). Na^+ current was inactivated by 10-ms prepulses to -45 mV. The voltage-clamp protocols are

illustrated in Figures 1 and 2. I_{TO1} amplitudes were measured as peak amplitudes minus steady-state values at the end of the test pulses (V_{test}). For I_{Kr} , we measured the tail currents on repolarization to -50 mV sensitive to almokalant (2 μ mol/L; specific I_{Kr} blocker).¹¹ For I_{Ks} , we measured the almokalant-insensitive tail currents on repolarization to -25 mV. For I_{K1} , we measured steady-state values at the end of V_{test} .

The standard-buffer solution used for the experiments was composed of (mmol/L): NaCl 145, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 1.0, glucose 11, HEPES 10, pH 7.4 with NaOH at 37°C. The patch-pipette solution contained (in mmol/L): potassium aspartate 125, KCl 20, $MgCl_2$ 1.0, MgATP 5, HEPES 5, EGTA 10, pH 7.2 with KOH.

The data are expressed as mean $\pm$ SEM. Intergroup comparisons were made with the Student's *t* test for unpaired and paired data groups, after testing for the normality of distribution. Differences were considered significant if $P < 0.05$.

Results

Action-Potential Characteristics

Typical examples of RV and LV M action potentials are shown in Figure 1. Quantitative data are given in the Table. RV M cells had a more pronounced spike-and-dome configuration than LV M cells at fast and slow pacing rates.¹ Both the action-potential upstroke and plateau (phase-0 and phase-2 amplitude) were larger in LV M cells, but only at fast rates. On average, action potentials were of shorter duration in RV than LV, and they displayed less prolongation (absolutely as well as relatively) upon increasing the CL from 500 to 4000 ms.

Properties of I_{TO1}

I_{TO1} activated at $V_{test} \geq -20$ mV in both ventricles, but amplitudes were significantly larger in RV than LV at all V_{test} (Figures 1 and 2A). 4-Aminopyridine (5 mmol/L) nearly completely suppressed I_{TO1} in both cell types. Inactivation during the V_{test} was best fitted with a single exponential function yielding similar time constants for RV and LV. The voltage dependence of I_{TO1} steady-state inactivation (Figure 2B) was well described by a Boltzmann fit with half points ($V_{0.5}$) of -52 ± 0.6 and -50 ± 0.5 mV, and slope factors of 6.8 ± 0.6 and 4.5 ± 0.5 mV in RV and LV, respectively ($P = NS$). Time-dependent recovery from inactivation was not different between the ventricles.

Figure 1. – K^+ currents I_{To1} and I_{Ks} are larger in RV than LV M cells isolated from the same normal dog heart, whereas I_{K1} is similar.

I_{To1} was activated by V_{test} from -40 up to +70 mV from a holding potential of -70 mV (interval 10 s). Shown are the traces of -10 to +70 mV. I_{Ks} was recorded in the presence of almokalant; we applied depolarizations from -20 up to +70 mV followed by repolarizations to -25 mV. Shown are the traces of -10, +10, +30, +50, and +70 mV. Holding potential was -50 mV (interval 20 s). I_{K1} was recorded during V_{test} of -20 to -140 mV. Shown are the traces of -70 to -140 mV. Holding potential was -50 mV (interval 3 s). Left horizontal bars indicate 0-pA level. Capacitances of the RV and LV M cells used for illustrations are similar (I_{To1} : 247 and 226 pF; both I_{Ks} and I_{K1} : 249 and 244 pF). Action potentials (microelectrode technique; vertical scale bars indicate 0 to -50 mV) were recorded at pacing CLs of 500 and 4000 ms, and illustrate that the difference of configuration and rate-dependent prolongation between the ventricles is likely related to underlying K^+ -current differences.

Figure 2. – Quantitative data for I_{TO1} , I_{Ks} , I_{Kr} , and I_{K1} in RV versus LV M cells from 9 dogs. Current density-voltage relation (**A**; $n_{RV}=12$, $n_{LV}=10$) and steady-state inactivation (**B**; $n_{RV}=6$, $n_{LV}=9$; 1-s conditioning pulses, 300-ms V_{test} , 10-s interval) for I_{TO1} . Activation for I_{Ks} (**C**) and I_{Kr} tail currents (**D**; $n_{RV}=18$ and $n_{LV}=13$). Current density-voltage relation for I_{K1} steady-state activation (**E**; $n_{RV}=8$ and $n_{LV}=5$). Asterisks indicate statistically significant differences in I_{TO1} and I_{Ks} current densities between RV and LV ($P<0.05$). cond indicates conditioning voltage.

Table. – Action-Potential Characteristics of Canine RV and LV M Cells

	RV M cells (n=11)	LV M cells (n=15)
Pacing CL 500 ms		
Amplitude, mV		
Phase 0	118 ± 2*	123 ± 2
Phase 1	92 ± 5*	105 ± 3
Phase 2	102 ± 2*	108 ± 2
Resting membrane potential, mV	-84 ± 2	-84 ± 1
APD ₉₅ , ms	272 ± 8*	294 ± 7
APD ₅₀ , ms	206 ± 7*	236 ± 6
Pacing CL 4000 ms		
Amplitude, mV		
Phase 0	113 ± 2	118 ± 2
Phase 1	71 ± 6*	87 ± 4
Phase 2	102 ± 2	105 ± 2
Resting membrane potential, mV	-84 ± 2	-84 ± 1
APD ₉₅ , ms	366 ± 17 (+35%)*	437 ± 34 (+49%)
APD ₅₀ , ms	297 ± 13 (+44%)*	379 ± 32 (+61%)

Microelectrode technique. Cells were obtained from 8 dogs. Amplitudes were measured as the voltage difference between resting membrane potential and peak of upstroke (phase 0), deepest level of notch (phase 1), and dome (phase 2). Action-potential duration was measured at 95% (APD₉₅) and 50% of repolarization (APD₅₀). Percentages in brackets indicate relative increase vs CL 500 ms. **P* < 0.05 vs LV M cells.

Properties of I_{Ks} and I_{Kr}

I_{Ks}-tail currents were evaluated on repolarization to -25 mV with I_{Kr} blocked by almokalant. Examples of current traces are shown in Figure 1. Pooled data are given in Figure 2C. There was no saturation of tail-current amplitudes. Voltage dependence of I_{Ks} activation was similar for both cell types, but density was significantly larger in RV: 0.72 ± 0.12 pA/pF than in LV: 0.38 ± 0.13 pA/pF (*P* < 0.05; depolarization to +50 mV). This difference persisted after increasing I_{Ks} in K⁺-free solution (0 [K⁺]_O): 0.98 ± 0.21 pA/pF in RV versus 0.58 ± 0.17 pA/pF in LV. Deactivation proved similar in RV and LV myocytes. Tail currents in 0 [K⁺]_O were best fitted by biexponential functions on repolarization to -10 to -40 mV, and by monoexponential functions on more negative repolarizations (-50 to -80 mV). At -20 mV, time constants of the fast and slow components were 228 ± 25 and 1105 ± 199 ms in RV (n=7) and 278 ± 35 and 1486 ± 269 ms in LV (n=6); at -60 mV, monoexponential time constants were 99 ± 16 in RV and 94 ± 11 in LV (*P* = NS for all).

I_{Kr} was quantified as the almokalant-sensitive tail-current portion measured by digital subtraction at -50 mV in 4.0 mmol/L [K⁺]_O (Figure 2D). Activation showed saturation at conditioning voltages > +20 mV. Boltzmann fits to the data revealed V_{0.5} of 2.9 ± 1.0 and 4.3 ± 2.5 mV in RV and LV, respectively, while corresponding slope fac-

tors were 6.2 ± 2.1 and 5.3 ± 0.8 mV ($P = \text{NS}$). I_{K_r} density was not different between RV and LV M cells. Voltage dependence and time course of I_{K_r} deactivation were also not different.

Properties of I_{K1}

Whole-cell recordings of I_{K1} are shown in Figure 1. I_{K1} rapidly activated and showed inactivation at the more negative voltages. In all cases, this current was fully inhibited in 0 $[K^+]_O$. There were no differences in the magnitude of I_{K1} (initial minimal values as well as steady-state levels) between RV and LV throughout the voltage range tested (Figure 2E).

Discussion

For interventricular comparisons of action potentials and K⁺ currents, we isolated myocytes from the deep subepicardial layers of the RV and LV free wall of the same canine hearts. In both ventricles, these myocytes have been designated M cells on the basis of distinctive electrophysiological characteristics.^{1,3-6} Our results show that action potentials have a deeper notch, a shorter duration and less prolongation upon slowing of the pacing rate in RV compared to LV M cells. A longer APD in the LV versus RV has already been recorded in dogs, both in vitro^{1,8} and in vivo (in dogs with complete atrioventricular block).⁹ In 6 dogs with sinus rhythm (CL: 507 ± 32 ms), we found endocardial monophasic APDs to be longer in LV than RV in all animals: 219 ± 6 versus 203 ± 6 ms ($P < 0.05$).¹² Taken together, these data indicate that a larger LV than RV APD exists at normal heart rates and during bradycardia.

The presence of I_{K_r} and I_{K_s} was confirmed in M cells from the LV and demonstrated also in RV M cells. Densities of I_{K_r} were similar in both ventricles. I_{K_s} density however, was significantly larger in RV and this difference could explain, at least partly, why APD₅₀ and APD₉₅ were longer and why the APD/pacing-CL relationship was steeper in LV than in RV M cells. Heterogeneity of I_{K_s} across the transmural LV wall has been linked to dispersion of repolarization and the danger of torsades de pointes.^{4,6} Our results on I_{K_s} (and I_{TO1}) suggest that arrhythmogenic electromotive gradients could also arise at the septal junction of the RV and LV.

In human ventricular myocytes, the presence of I_{K_r} and I_{K_s} has also been demonstrated.¹³ Interestingly, Li et al¹³ made their observations in apparently undiseased RV myocytes of patients with left-sided heart failure. The finding of substantial amplitudes of I_{K_r} and I_{K_s} , as well as the sensitivity of both components to their blockers E-4031 and indapamide, may underscore the importance of these currents for human ventricular repolarization, as expected from the clinical response to class Ia and class III agents in patients, and from molecular studies on K⁺ channels in human myocardial tissue.

Our finding of a large I_{TO1} in RV M cells is in keeping with the prominent spike-and-dome morphology of the action potentials. Yan and Antzelevitch¹⁴ pre-

sented evidence that the distribution of I_{TO1} across the canine ventricular wall is causally linked to the J wave of the ECG. The joint results of this and another study⁸ indicate that a large I_{TO1} -mediated notch can be found throughout most of the RV mass, which suggests that the contribution of the RV to the formation of the J wave on the ECG may be larger than previously assumed. Furthermore, this may have important consequences for our understanding of the Brugada syndrome. ST-segment elevation in the right precordial ECG leads of patients suffering from this disorder has been linked to the concept of 'all-or-none repolarization' in the RV epicardium.¹⁵ If our data are applicable to patients, then the substrate predisposed to all-or-none repolarization may cover most of the RV transmural wall.

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Cellular Basis of Biventricular Hypertrophy and Arrhythmogenesis in Dogs with Chronic Complete Atrioventricular Block and Acquired Torsade de Pointes

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Abstract

Background: In the dog with chronic complete atrioventricular block (AVB) torsade-de-pointes arrhythmias (TdP) can be induced reproducibly by class-III antiarrhythmic agents. In-vivo studies reveal important electrophysiological alterations of the heart at 5 weeks of AVB resulting in increased proarrhythmia. Autopsy studies indicate the presence of biventricular hypertrophy. In this study, the cellular basis of proarrhythmia and hypertrophy in chronic AVB was investigated.

Methods and Results: From chronic-AVB dogs with increased heart weights and TdP, left midmyocardial and right ventricular myocytes were isolated by enzymatic dispersion. These myocytes were significantly larger than sinus-rhythm (SR) controls. In chronic AVB the action-potential spike-and-dome configuration was preserved. However, the action-potential duration (APD) at 95% and 50% of repolarization of the left midmyocardium was significantly larger in chronic AVB than in SR, with little change in the right ventricle, causing enhanced interventricular dispersion of repolarization at slow pacing rates. Treatment with the class-III agent almokalant increased the APD to a much larger extent in chronic-AVB than in SR myocytes and resulted in a higher incidence of early afterdepolarizations (EAD). EAD had their take-off potential between -35 and 0 mV. There was no evidence that spontaneous sarcoplasmic reticulum Ca^{2+} release underlies these EAD.

Conclusions: In the dog, chronic AVB leads to hypertrophy of both right and left ventricular myocytes. The repolarization abnormalities predisposing for class-III dependent TdP in vivo are the result of cellular electrophysiological remodeling.

Introduction

The dog with chronic complete atrioventricular block (AVB) has been described as an animal model of acquired torsade-de-pointes arrhythmias (TdP).^{1,2} Clinically relevant doses of class-III antiarrhythmic agents, either alone (spontaneous TdP) or in combination with programmed electrical stimulation, can evoke such a proarrhythmic response in a reproducible manner in the majority of anesthetized dogs with chronic AVB.^{2,3} In-vivo studies show that the duration of AVB is an important determinant of the susceptibility to acquired TdP, because the latter are rarely inducible at 0 weeks of AVB (acute AVB) or at sinus rhythm (SR), but are readily inducible at 5 weeks or later (chronic AVB) in most animals.⁴ The increased susceptibility to arrhythmias in chronic AVB has been related to an inhomogeneous prolongation of the monophasic ventricular action potential (in the left ventricle more than the right ventricle), leading to enhanced regional dispersion of repolarization.⁴ Furthermore, class-III dependent early afterdepolarizations (EAD) are prominent, which may explain the more frequent observation of ventricular ectopic beats^{5,6} in chronic-AVB dogs.⁴

Structural adaptations accompany the altered hemodynamic load in the heart with AVB. Autopsy studies have demonstrated increased heart-weight to body-weight ratios with significant contributions of both the right- and left-ventricular mass.⁴ Morphologically, the biventricular hypertrophy is characterized by an eccentric expansion with increased right- and left-ventricular diameters, as seen during volume overload.

Therefore, proarrhythmia in dogs with chronic AVB seems to be based upon electrophysiological remodeling in the presence of bradycardia and cardiac hypertrophy. This study was designed to investigate the cellular basis of cardiac electrophysiological and structural changes in this animal model and to assess their contribution to the facilitation of TdP induction.

Methods

The animal experiments were conducted in accordance with the guidelines of the American Physiological Society and under the regulations of the Committee for Experiments on Animals of the Maastricht University, The Netherlands.

In-Vivo Studies

Adult mongrel dogs of either sex and weighing between 22 and 32 kg were used for the experiments. AVB was created according to the procedure described by Steiner and Kovalik⁷ ($n_{\text{dogs}}=9$). For a complete description of the perioperative care we refer to earlier publications.²⁻⁴

To document the induction of TdP, the class-III agent almokalant was administered intravenously at a concentration of 0.12 mg/kg/10 min infusion time in 7 of the 9 animals with chronic AVB.⁸ Almokalant is a known inhibitor of the rapidly-activating

component (I_{Kr}) of the delayed-rectifier K^+ current (I_K)⁹ and increases regional dispersion of repolarization.^{8,10} Surface ECG leads and right- and left-ventricular monophasic-action-potential catheters were positioned for simultaneous on-line recording of the signals. TdP was defined as a polymorphic ventricular tachycardia consisting of ≥ 5 beats twisting around the baseline in a setting of prolonged QT(U) duration. These experiments were performed during anesthesia at the latest 1 week before the dogs were sacrificed for cell isolation. In subsequent cellular experiments allopurinol was also used. Thus, the response to class-III treatment was tested both in vivo and in vitro in dogs with documented episodes of TdP and increased heart weights.

Cell-Isolation Procedure

Anesthetized chronic-AVB ($n_{\text{dogs}}=9$) and SR dogs ($n_{\text{dogs}}=9$; similar body weights) received 10,000 IU heparin IV on thoracotomy. In the former group AVB had been present for a duration of 6 ± 1 weeks (range, 5 to 9 weeks). The hearts were excised and washed in a cardioplegic solution at 5 to 10°C. Single right and left ventricular myocytes were isolated simultaneously according to a procedure adapted from Powell.¹¹ Cannulas were quickly inserted in and sutured to the left-anterior-descending and right coronary arteries under continuous perfusion with cardioplegic solution. Subsequent perfusion was done at 37°C with: (1) nominally Ca^{2+} -free standard-buffer solution for 10 min, (2) Ca^{2+} -free solution with collagenase for 10 min, and (3) Ca^{2+} -free solution with collagenase plus protease for 20 to 30 min. The epicardial surface temperature and the pH of the cardiac effluent were regularly measured to check for an adequate perfusion of the tissues. Enzymes were washed out with 0.2 mmol/L [Ca^{2+}] standard-buffer solution for 10 min. Finally, the tissue was minced and the cell suspension filtered and washed. Left ventricular midmyocardial myocytes were isolated by careful harvesting of the middle third of the transmural wall of the perfused wedge, as described earlier.¹² Right ventricular myocytes were harvested as a mixture from the transmural wall. Isolated myocytes were stored at room temperature in standard-buffer solution.

Experimental Set-Up

A sample of the cell suspension was transferred to a perfusion chamber (0.5 mL) and superfused with standard-buffer solution at a rate of 3 mL/min and at $37 \pm 0.5^\circ\text{C}$. Myocytes used for the experiments were selected on the basis of the following criteria: having sharp striations, clear contours, transparent cytoplasm without granulations or blebs, and a resting membrane potential below -75 mV.

Myocytes were imaged by a video camera connected to the inverted microscope (Diaphot 300, Nikon, Inc) of the set-up. The length and width of each cell were measured on a defined area of the monitor with a $\times 40$ microscopic objective. Although the width was not constant along the length of many cells, we measured this dimension as the estimated average of the broadest and thinnest part of a cell near its middle. More than 600 left midmyocardial and more than 400 right ventricular cells per group were measured.

For the experiments with microelectrodes, sharp standard glass electrodes filled with 3.0 mol/L KCl (resistance 30 to 60 M Ω) were used. Action potentials were recorded with a microelectrode amplifier (Axoclamp-2B, Axon Instruments, Inc) at cycle lengths (CL) of 300, 400, 500, 1000, 2000, 3000 and 4000 ms. The values of action-potential parameters at baseline and during treatment with almokalant presented throughout the text are the averaged measurements of five beats during steady-state pacing. Myocyte contractions were recorded with a video-edge-motion detector (Crescent Electronics) at 16-ms temporal resolution.¹³ The analog output signals were digitized and stored on computer hard disk. Cell shortening was expressed in percent: amplitude in micrometers relative to cell length.

For the measurements of intracellular Ca²⁺ transients and action potentials, the whole-cell variant of the patch-clamp technique was used.¹⁴ Pipettes were pulled from borosilicate glass and had resistances of 1.0 to 3.0 M Ω when filled with 140 mmol/L KCl. Membrane potentials were recorded in the current-clamp mode (Axopatch-1D, Axon Instruments, Inc). The data were filtered at 5 kHz, read into a personal computer through an analog-to-digital converter (2 kHz) and stored for later analysis. The data acquisition program also controlled the command potential and various components of the intracellular-Ca²⁺ ([Ca²⁺]_i) measurement system (Fastlab Software, Indec Systems). The pipette solution contained Fluo-3 and Fura-red (Molecular Probes).¹⁵ The combined use of these fluorescent indicators was validated in confocal microscopy.¹⁶ Our experimental set-up for [Ca²⁺]_i measurements has been described elsewhere.¹⁵

Solutions

The standard-buffer solution contained (in mmol/L): NaCl 145, KCl 4.0, CaCl₂ 1.8, MgCl₂ 1.0, Na₂HPO₄ 1.0, glucose 11, HEPES 10, pH 7.4 with NaOH at 37°C and bubbled with O₂. In the cold cardioplegic solution, KCl was set to 8.0 mmol/L. For the cell isolation, collagenase (1.1 mg/mL; Type A, Boehringer Mannheim) and protease (0.05 mg/mL; Type XIV, Sigma Chemical Co) were used in the presence of bovine serum albumin (1.0 mg/mL). The patch-pipette solution contained (in mmol/L): K⁺ aspartate 120, KCl 20, MgCl₂ 0.5, MgATP 5, NaCl 10, HEPES 10, Fluo-3 0.03, Fura-red 0.07, pH 7.2 with KOH. All of the chemicals were reagent grade and cell-culture tested (purchased from Sigma Chemical Co). Almokalant was kindly provided by Drs. L. Carlsson and G. Duker, Astra Hässle, Mölndal, Sweden.

Statistical Analysis

The data are expressed as means $\pm$ SD. Intergroup comparisons were made by the Student's *t* test for unpaired (chronic AVB vs SR; right vs left ventricle) and paired (baseline vs treatment) data groups, respectively, after testing for the normality of distribution. The χ^2 test was used when the data were presented as a proportion. Differences were considered significant if *P*<0.05.

Results

In-Vivo Studies

In our companion study⁴ we report on the electrophysiological consequences of clinically relevant doses of *d*-sotalol in dogs with AVB. In preliminary cellular experiments of the present study, we chose to use almokalant because this agent exhibits a high specificity of I_{K_r} inhibition at nano- to micromolar concentrations,⁹ whereas *d*-sotalol may also exert other actions.^{17,18} Accordingly, the proarrhythmic potential of almokalant was tested in vivo ($n_{\text{dogs}}=7$). An example of the electrophysiological consequences of almokalant treatment and subsequent spontaneous TdP induction is shown in Figure 1. At 4 ± 2 weeks of AVB, the class-III agent increased the QT interval from 415 ± 90 ms to 545 ± 105 ms ($P<0.05$) without significant change of the CL of the idio-ventricular rhythm (from 1545 ± 300 ms to 1655 ± 295 ms; $P=NS$). Almokalant caused an increase of the monophasic action-potential duration (APD) in the left ventricle from 380 ± 55 ms to 525 ± 115 ms ($P<0.05$) and in the right ventricle from 335 ± 65 ms to 425 ± 110 ms ($P<0.05$), contributing to an enhanced interventricular dispersion of repolarization of 45 ± 15 ms at baseline to 100 ± 50 ms during treatment ($P<0.05$). In 6 of the 7 dogs, TdP ensued spontaneously, while in the remaining animal TdP occurred neither spontaneously nor with programmed electrical stimulation.

Cellular Basis of Biventricular Hypertrophy

When weighed directly after excision, the hearts of chronic-AVB dogs ($n_{\text{dogs}}=9$; 6 ± 1 weeks of AVB) were significantly heavier than those of SR controls ($n_{\text{dogs}}=9$): 285 ± 25 g vs 222 ± 59 g, respectively ($P<0.05$). When corrected for body weight, the difference in heart weight remained significant: 10.3 ± 1.3 g/kg vs 8.5 ± 1.5 g/kg, respectively ($P<0.05$). Myocytes were successfully isolated from the right ventricular free wall and the left ventricular midmyocardium of all dogs. Representative photomicrographs of single left midmyocardial cells are shown in Figure 2. Right ventricular myocytes showed a similar morphology, both in chronic AVB and in SR. All cells were quiescent during superfusion with the standard-buffer solution containing 1.8 mmol/L $[Ca^{2+}]$. In the SR group, right ventricular myocytes were of equal length and width as compared to left midmyocardial myocytes: cell length was 140 ± 10 μm and 140 ± 16 μm ($P=NS$), and cell width was 24 ± 2 μm and 25 ± 2 μm , respectively ($P=NS$). In the chronic-AVB group, myocytes from both ventricles were significantly longer than SR controls, being 172 ± 8 μm (right ventricle) and 158 ± 7 μm (left midmyocardium; both $P<0.05$, chronic AVB vs SR). The difference in cell length of right vs left ventricle in chronic AVB was statistically significant ($P<0.05$). By contrast, the width of these cells was not different from SR controls: 26 ± 1 μm and 26 ± 2 μm , right vs left ventricle, respectively ($P=NS$). Frequency distributions for cell length and width are shown in Figure 3.

Figure 1. – Two runs of spontaneous TdP in a dog with 3 weeks AVB upon the administration of almokalant (0.12 mg/kg/10 min infusion time).

Shown are the electrocardiographic leads I, II and AVR, and the monophasic-action-potential recordings from the left (LV MAP) and right ventricular endocardium (RV MAP). At baseline (1 CONTROL) there is an idioventricular rhythm with a CL of 1190 ms and a QT time of 375 ms. At that moment the left ventricular APD is 335 ms vs 290 ms in the right ventricle. During almokalant treatment (2 ALMOKALANT) both the left and right ventricular action potentials prolong to approximately 445 ms and 350 ms. This inhomogeneous action-potential prolongation contributes to an enhancement of the interventricular dispersion of repolarization from 45 ms at baseline to approximately 95 ms during treatment. Short runs of spontaneous TdP were present until >20 min after the end of the almokalant infusion. Characteristically, the initiating ventricular complexes occur before full termination of the T wave of the ECG and simultaneously with EAD and triggered action potentials, as recorded by the monophasic-action-potential catheters in both the right and left ventricle. For an estimation of the left ventricular APD during almokalant treatment, the descent of the repolarization is drawn artificially.

Action Potentials in Single Myocytes from Dogs with Chronic AVB

Action potentials were recorded at various physiologically relevant pacing CL with the microelectrode technique in a total of 51 myocytes (chronic AVB: $n_{\text{dogs}}=7$, $n_{\text{cells,LV}}=13$, $n_{\text{cells,RV}}=9$; SR: $n_{\text{dogs}}=8$, $n_{\text{cells,LV}}=15$, $n_{\text{cells,RV}}=14$). Representative action potentials of the four different cell groups are shown in Figure 4. In SR, the spike-and-dome configuration was more pronounced in right-ventricular than left-midmyocardial action potentials and more pronounced at longer CL, as expected. In chronic AVB, this pattern was preserved or slightly accentuated (Figure 4).

Figure 2. – Photomicrographs of single myocytes isolated from the left ventricular midmyocardium of a chronic-AVB (upper panel) and a SR dog (control; lower panel). The chronic-AVB myocytes are larger than their SR controls with cell lengths of approximately 230 μm (upper panel, cell on the right) and 160 μm (upper panel, cell on the left). Quantitative data for mean myocyte lengths and widths are presented in the Results section. The 50- μm calibration bar applies to both panels. Magnification $\times 1000$.

Figure 3. – Frequency distributions for lengths and widths of right ventricular free wall and left midmyocardial myocytes in chronic-AVB and SR groups. In total, 452 and 403 right, and 607 and 676 left ventricular cells are measured in chronic AVB and SR, respectively. A relatively higher number of long cells ($\geq 150 \mu\text{m}$) can be observed in chronic AVB.

Figure 4. – Action potentials of single myocytes from dogs with chronic AVB as a function of pacing CL.

In each panel, pacing CLs are 500, 2000 and 4000 ms from left to right, respectively. Action potentials of left midmyocardial myocytes are more prolonged in chronic AVB than SR. The APD is larger in the left than the right ventricular cells in both conditions, especially at long CL. Microelectrode technique.

In SR, the APD at 95% of repolarization (APD_{95}) increased on slowing of the pacing rate, as expected. Pooled data are shown in Figure 5. Chronic AVB significantly steepened this APD_{95}/CL relationship in the left midmyocardial myocytes ($P < 0.05$, chronic AVB vs SR), but not in the right ventricular cells ($P = NS$, chronic AVB vs SR). The interventricular difference of APD_{95} was therefore significant at all CL (Figure 5). The same was found for the APD at 50% of repolarization (APD_{50}). This parameter increased to the same extent as the APD_{95} in chronic AVB, again with significant changes in the left midmyocardial, but not the right ventricular cells. The maximal velocity of repolarization during phase 3 (measured as the most negative first derivative of the membrane potential in that phase) was not different between chronic AVB and SR in both ventricles (data not shown).

In an additional population of 32 myocytes of the same dogs we recorded action potentials during whole-cell patch clamp (chronic AVB: $n_{\text{cells,LV}}=7$, $n_{\text{cells,RV}}=8$; SR: $n_{\text{cells,LV}}=10$, $n_{\text{cells,RV}}=5$). We confirmed the differences between APD of left vs right ventricle, and chronic AVB vs SR under otherwise similar conditions (data not shown).

Figure 5. – Pooled data of APD₉₅ as a function of pacing CL using the microelectrode technique.

In chronic AVB, action potentials of left midmyocardial myocytes are significantly longer than of right ventricular myocytes (left panel; *, $P < 0.05$). Also, the average APD₉₅ of chronic-AVB left midmyocardial cells is larger than of SR controls (left vs right panel; +, $P < 0.05$).

We also examined the peak contraction amplitudes and $[Ca^{2+}]_i$ accompanying the action potentials. At the pacing CL of 1000 ms the peak contraction amplitude measured $4.3 \pm 1.9\%$ vs $3.1 \pm 3.7\%$ in right ventricular cells ($P = NS$), and $7.2 \pm 2.2\%$ vs $5.1 \pm 2.1\%$ in left midmyocardial myocytes ($P < 0.05$, chronic AVB vs SR, respectively). Contraction durations were not different in these cell groups. $[Ca^{2+}]_i$ peaked at 460 ± 171 nmol/L vs 517 ± 102 nmol/L in the right ventricular mixture ($P = NS$, chronic AVB vs SR, respectively), and at 457 ± 146 nmol/L vs 496 ± 107 nmol/L in the left midmyocardium ($P = NS$).

Increased Sensitivity of Chronic-AVB Myocytes to Almokalant

We treated both SR and chronic-AVB myocytes with 1 $\mu\text{mol/L}$ almokalant. A significant prolongation of the action potential was observed in all cells tested ($n = 29$; microelectrode technique), which was most pronounced at long pacing CL. Representative examples of action potentials of chronic-AVB myocytes during treatment with almokalant are shown in Figure 6. In chronic-AVB cells the relative increase of the APD₉₅ during almokalant (in the absence of EAD!) was much larger than in SR, independent of the chamber. This is illustrated by the pooled data of APD₉₅ shown in Figure 7A. In all cells, the APD₅₀ increased to the same extent as the APD₉₅.

In the myocytes studied with patch electrodes ($n = 32$), we found comparable responses to almokalant; likewise, the average increase of the APD was much larger in the chronic-AVB than the SR cells (Figure 7B).

Action-potential prolongation was often followed by the occurrence of EAD in chronic-AVB myocytes, but not in SR cells, with the microelectrode as well as the

Figure 6. – Action-potential prolongation by almokalant in a left midmyocardial and a right ventricular myocyte during chronic AVB.

The thin dashed action potentials are from the myocytes under baseline conditions and the thick solid action potentials during treatment with the class-III agent. Action-potential prolongation is most pronounced at long pacing CL and, in this case, more in the left than the right ventricle. In the upper panel, an example of an EAD is shown.

patch-electrode technique. The Table expresses this increased sensitivity as a larger proportion of cells in which EAD were observed during treatment with almokalant. EAD were generated at the longer pacing CL of ≥ 2000 ms. No EAD were observed at CL of ≤ 500 ms. Whenever EAD appeared they caused a significant increase of the APD. In the most sensitive cells plateau arrests were often observed, ie, prolonged phases of de-

polarization (sometimes lasting several seconds) that were followed by one or more EAD before a rapid repolarization. Large beat-to-beat variability of the APD often characterized the class-III action, as is shown in Figure 8.

Characteristics of the Almkolant-Induced EAD in Chronic AVB

The take-off potentials of almkolant-induced EAD in chronic AVB ranged between -35 and 0 mV. On average, EAD started at significantly more negative levels in the right ventricle ($n_{\text{EAD}}=50$) than the left midmyocardium ($n_{\text{EAD}}=100$): -30 ± 2 mV vs -19 ± 5 mV, respectively ($P < 0.05$), whereas the action potentials analyzed for this purpose had equal resting membrane potentials. EAD amplitudes were larger in right vs left ventricular myocytes: 21 ± 6 mV vs 10 ± 5 mV ($P < 0.05$).

We addressed the question of whether spontaneous Ca^{2+} release from the sarcoplasmic reticulum could underlie these EAD, as described earlier for isoproterenol-induced EAD.¹⁹ Figure 9 illustrates typical findings for two left midmyocardial myocytes. $[\text{Ca}^{2+}]_i$ rose rapidly on depolarization, reflecting Ca^{2+} release from the sarcoplasmic reticulum. This release was followed by a rapid but incomplete decline of $[\text{Ca}^{2+}]_i$, and $[\text{Ca}^{2+}]_i$ typically remained elevated at approximately 30% above baseline values as long as the membrane potential did not recover completely (Figure 9, left panel A). EAD during this phase, however, were not associated with distinct new $[\text{Ca}^{2+}]_i$ transients. Small fluctuations of $[\text{Ca}^{2+}]_i$ were sometimes observed, in amplitude always less than 5% of the initial $[\text{Ca}^{2+}]_i$ transient. In the case of cell shortening the normal twitch contraction was followed by a relaxation phase during which cell length attained near-resting levels. However, as with the $[\text{Ca}^{2+}]_i$, full relaxation awaited full repolarization of the action potential (Figure 9, right panel B). In a few cells and only when large-amplitude EAD were generated, we could discern small early aftercontractions that followed the EAD upstroke with a delay of several tens of milliseconds (Figure 9B, arrow). Similar results on $[\text{Ca}^{2+}]_i$ and cell shortening during EAD were obtained in 30 cells treated with almkolant.

Discussion

Cellular Hypertrophy in Chronic AVB

The present study confirms the earlier finding of increased heart-weight to body-weight ratios in adult dogs with chronic AVB.⁴ The hearts are enlarged consistent with eccentric hypertrophy, as described for volume overload.²⁰ The hypertrophy is related to increased lengths of both right and left ventricular myocytes. Myocyte growth has been observed in most large animal models of cardiac hypertrophy reported in the literature (eg, References 21 through 23). We found that the biventricular hypertrophy of chronic AVB was not a homogenous process: growth responses were larger in the right than left ventricular myocytes (+23% vs +13% for cell length, respectively), supporting the autopsy finding of a larger relative increase of the right- than the left-

Figure 7. – Pooled data of APD₉₅ (in action potentials without EAD) as a function of pacing CL during treatment with almokalant. **A**, Data obtained using the microelectrode technique. **B**, Patch-electrode technique. Almokalant caused a significant increase of the APD₉₅ in all cell groups, regardless of the technique used. In absolute terms, this was much more pronounced and within a broader range during chronic AVB than in SR. *, *P* < 0.05 between chronic AVB and SR.

Table. – Proportion of Cells Generating EADs During Almokalant Treatment

	Pacing CL	
	2000 ms	4000 ms
Chronic AVB		
Left Midmyocardium	10/18 *	15/18 *
Right Ventricular Free Wall	7/14 *	9/14 *
Control (Sinus Rhythm)		
Left Midmyocardium	0/16	3/16
Right Ventricular Free Wall	3/14	4/14

Values are cell numbers. Almokalant, 1 $\mu\text{mol/L}$. *, $P < 0.05$ between Chronic AVB and Sinus Rhythm at that CL.

Figure 8. – EADs during treatment with almokalant: beat-to-beat differences of the APD. Shown is a continuous recording of eight action potentials at a pacing CL of 1000 ms. In the first beat a plateau arrest can be observed that lasts for the full CL and is overcome only after the pacing stimulus of the next action potential. In consecutive beats, the APD_{95} varies from 500 to 900 ms. This is an example of temporal dispersion of repolarization within a single cell.

ventricular weight.⁴ This differential ventricular growth response resembles the finding in another animal model of biventricular hypertrophy related to volume overload (aortocaval fistula).²² Also, in pigs with heart failure due to rapid pacing, myocytes were larger in the right than left ventricle.²⁴ The preponderance of right ventricular cell growth in chronic AVB may reflect a greater impact of the altered hemodynamics on that chamber after the transition from SR to idioventricular rhythm.

As to the functional characteristics of chronic-AVB myocytes, we demonstrated that peak amplitudes of contraction and $[\text{Ca}^{2+}]_i$ at 1000 ms pacing CL were not different from SR cells (or, if anything, were slightly increased). These data, which are the subject of further investigations, provide additional support for the notion, based on in-vivo hemodynamic measurements,⁴ of a compensated form of cardiac hypertrophy.

Figure 9. – Cellular handling of $[Ca^{2+}]_i$ during class-III dependent EADs in chronic AVB. In the left half of this figure (panel **A**), the simultaneous recording of an action potential and $[Ca^{2+}]_i$ is depicted using current clamping in the whole-cell variant of the patch-clamp technique and with the fluorescent indicators Fluo-3 and Fura-red in the internal pipette solution. In the right half (panel **B**), the action potential (microelectrode technique) is recorded simultaneously with cell shortening. In both panels, left ventricular midmyocardial cells were treated with almokalant at 1 μ mol/L during stimulation at a pacing CL of 4000 ms, leading to the generation of EAD. Full relaxation of the $[Ca^{2+}]_i$ and contraction, in **A** and **B**, respectively, was delayed until full repolarization of the action potential. Distinct or high-amplitude peaks of $[Ca^{2+}]_i$ (ie, Ca^{2+} aftertransients) were *not* observed during the EAD, as illustrated in **A**. Small aftercontractions following the upstroke of the EAD were rarely observed. An example is shown in **B** (arrow).

Cellular Electrophysiological Remodeling in Chronic AVB

Our data show that myocytes from dogs with chronic AVB have: (1) an intrinsic disturbance of repolarization with prolonged action potentials at baseline, (2) interventricular differences of repolarization, and (3) an increased susceptibility to class-III antiarrhythmic agents.

Intrinsic disturbance of repolarization. Prolongation of the left-ventricular action potential is a consistent observation in myocardial hypertrophy of different causes in several species.^{25–27} Cellular electrophysiological data on right-ventricular hypertrophy are scarce. In pressure-overload induced right-ventricular hypertrophy, increases of the APD have been reported.²⁸ Many of the sarcolemmal ion channels, exchangers and pumps, as well as intracellular ion transporters that shape the action potential under normal conditions can show functional defects leading to delayed repolarization in hypertrophy.^{29,30} Other investigators have shown that this action-potential prolongation predisposes to an increased susceptibility to EAD³¹ and ventricular tachyarrhythmias.^{32,33} As yet, the ionic basis of action-potential changes in chronic AVB remains to

be elucidated. The APD_{95} and APD_{50} increased to a similar extent, indicating that the delay in repolarization was likely due to a disturbance at the plateau level. From the data in this study a prominent role for the transient outward current (I_{TO}) appears unlikely: the spike-and-dome configuration of the action potential was preserved or, if anything, accentuated. In this respect the dog with chronic AVB differs from the dog with pacing-induced heart failure.³⁴ In that model, downregulation of I_{TO} is at least partly responsible for an attenuation of the notch of the action potential and an increase of the APD,³⁴ which has been linked to the generation of lethal ventricular arrhythmias.³⁵ Additional insights into the ionic mechanisms of action-potential prolongation in chronic AVB come from the experiments with almokalant. Carmeliet⁹ demonstrated that in rabbit ventricular myocytes I_{Kr} is fully blocked by 1 $\mu\text{mol/L}$ of the agent and we have confirmed this in normal dog ventricular myocytes (data not shown). After block of I_{Kr} during almokalant treatment, we found that the APD was much larger in chronic-AVB left and right ventricular myocytes than in SR controls (Figure 7). This implies that ionic currents other than I_{Kr} contribute to the abnormal repolarization of chronic AVB, even though we cannot exclude that I_{Kr} also plays a role.

Interventricular differences of repolarization. To the best of our knowledge this is the first study to compare action-potential characteristics of single hypertrophied myocytes isolated from both the right and left ventricle of the same dog. Interventricular differences of the action potential are known to exist in the normal myocardium of dogs with SR^{6,36} and this was confirmed by the present study. In chronic AVB, we found larger APD in left midmyocardial than right ventricular myocytes at baseline, in contrast to the larger degree of hypertrophy in the latter than the former cells. Yet, the administration of almokalant increased the APD to a similarly high level in both ventricles. Thus, the presence of intrinsic repolarization disturbances was unmasked in right ventricular myocytes. At least three explanations could account for the interventricular differences of repolarization at baseline: (1) there was a different electrophysiological remodeling in both ventricles, (2) the charge carriers involved were the same, but their functional alterations mounted to different amplitudes, or (3) a finer balance of currents existed during the plateau phase because of a decrease in outward current and/or an increase in inward current.

Increased susceptibility to almokalant. Chronic-AVB cells showed an increased susceptibility to action-potential prolongation and EAD generation during class-III treatment. These EAD were observed at long pacing CL and had take-off membrane potentials ranging between -35 and 0 mV. Very small changes in the balance of inward and outward currents during the high resistance plateau can determine the appearance or absence of EAD. At least two possibilities could explain the increased number of EAD found in this study: (1) they occurred because the APD was initially prolonged, and/or (2) the charge carrier(s) responsible for their generation were more prominent in myocardial hypertrophy, eg, increased L-type Ca^{2+} current (I_{CaL}) or more spontaneous sarcoplasmic reticulum Ca^{2+} release. If the former is true, it follows that any intervention causing the action potential to shorten, would lead to the diminished appearance of

EAD. Preliminary results with the agent levcromakalim, an I_{K-ATP} activator, support this hypothesis: we found that in consecutive action potentials with EAD levcromakalim initially caused a decrease of the APD, followed by the disappearance of the EAD and a further shortening of the action potential. The finding that DAD remained also absent during this action-potential shortening was an argument against the possibility of spontaneous sarcoplasmic reticulum Ca^{2+} release to underlie the class-III dependent EAD of chronic AVB. Indeed, the characteristics of cytoplasmic Ca^{2+} during the EAD confirmed this notion. We did find that the relaxation of $[Ca^{2+}]_i$ and cell shortening was slow during action potentials with EAD. The decline of $[Ca^{2+}]_i$ always paralleled the repolarization regardless of the APD, which suggests that the voltage-dependence of cellular Ca^{2+} extrusion was maintained as under normal conditions. Our results in chronic-AVB myocytes resemble those of cesium-chloride-induced EAD in normal ferret ventricular muscle³⁷ and class-III-induced EAD in normal rabbit ventricular myocytes.³⁸ Although our data support an important role for the initial action-potential prolongation, we cannot exclude the possibility that intrinsic changes of I_{CaL} may be involved in the increased number of EAD.

Our finding that EAD amplitudes were larger in right than in left ventricular myocytes could be related to the lower take-off potentials in the former cells. If indeed I_{CaL} is the major charge carrier of these EAD, its triggering at lower take-off potentials would likely lead to larger current amplitudes.

Cellular Basis of In-Vivo Proarrhythmia

Our data show that the interventricular differences of APD were most pronounced at the longer pacing CL of ≥ 2000 ms, measuring maximally 100 ms at baseline. Because in most cases the CL of the idioventricular rhythms of the dogs were >1500 ms, we consider the cellular findings relevant for the in-vivo situation and find them consistent, at least qualitatively, with the monophasic-action-potential findings in the same dogs before they were sacrificed. We do not believe that interventricular dispersion itself is the direct cause for the initiation of torsade de pointes, but because it is always present during arrhythmogenesis it probably reflects the existence of dispersion of more closely juxtaposed regions. When using almokalant in our experiments, we found that this agent promoted temporal heterogeneity of repolarization with varying APD and EAD that appeared in an on-and-off fashion in consecutive beats during steady-state pacing. Dispersion could be enhanced even more if the heterogeneity of cellular repolarization occurred out of phase in various regions of the myocardium. We have demonstrated such spatial dispersion in vivo.³

Another important factor involved in the occurrence of TdP is ventricular ectopy. In-vivo recordings in the dog with chronic AVB confirm the importance of ventricular ectopic beats (Figure 1),^{2,4} however until now their origin and mechanisms remain obscure. While others have demonstrated triggered activity upon EAD in Purkinje fibers during in-vitro conditions that mimic proarrhythmia in vivo,³⁹⁻⁴¹ it can be concluded from the present study that in single cells from the working myocardium

abnormal impulse generation is limited to EAD that do not trigger new action potentials in these same cells. This conclusion is based on our findings that the EAD always: (1) arose from a take-off membrane potential less negative than -35 mV (above the threshold for full activation of Na^+ current) and did not show a rapid upstroke as in normal action potentials; (2) had an amplitude <30 mV; (3) prolonged the repolarization, but did not take the shape of normal action potentials; (4) were incapable of inducing normal, if any, contractions; and (5) behaved differently from the EAD observed in Purkinje fibers or in ventricular myocytes with take-off levels closer to the resting membrane potential. Thus, the characteristics of these membrane responses do not match our definition of the ventricular action potential.

DAD-triggered action potentials or abnormal automaticity were also not observed in the chronic-AVB cells. This all may mean that the substrate for extrasystolic activity involved in the initiation of TdP is multicellular (eg, phase-2 reentry) or includes the Purkinje network. In addition, the experimental setup chosen for this study lacks many of the components affecting arrhythmogenesis *in vivo*, such as adrenergic agonists, stretch and sudden rate changes. For this reason the absence of spontaneous action potentials in the single myocyte does not exclude their generation *in vivo*.

Limitations of the Study

One of the aspects of this study was to compare the action-potential characteristics of myocytes from the left midmyocardium with those from the right transmural ventricular wall. One could argue that this comparison is not fully adequate given the findings made in tissue studies that the shape of the ventricular action potential and the CL dependence of its duration vary across the free wall of both chambers.⁴² Because of the thinness of the right ventricular wall we found it difficult to isolate the cells according to their transmural site of origin in that chamber. Nevertheless, at long pacing CL right ventricular myocytes consistently had a typical action-potential configuration that was different from that of the left midmyocardial cells. In addition, the range of APD measured in left and right ventricular cells, eg, at a CL of 2000 ms, showed an overlap of only 10% in chronic AVB and of 20% in SR. A 'bad pick' of only epicardial or endocardial cells from the right ventricular mixture might explain this finding, but this is unlikely. A more plausible explanation is that the APD differences of midmyocardial vs epi- and endocardial myocytes are much less in the right than the left ventricle. The comparison of right transmural vs left transmural ventricular myocytes (instead of right transmural vs left midmyocardial cells, as made now) would likely underestimate the interventricular dispersion of repolarization present in these hearts and was therefore not applied.

When we compared the action-potential characteristics of our right ventricular mixture with the data presented by Sicouri and Antzelevitch,⁴² we found that the majority of our cells (93% for SR and 67% for chronic AVB) had a steep APD/CL relationship, which would identify them as right ventricular midmyocardial cells.

Conclusions

We conclude that AVB of chronic duration leads to inhomogeneous hypertrophy of right and left ventricular myocytes. Cellular hypertrophy is associated with an intrinsic defect of repolarization in the right and left ventricle. APD differences between left midmyocardial and right ventricular myocytes cause an enhanced interventricular dispersion of repolarization, which is most pronounced at the longer pacing CL. Almodant increases the APD to a much larger extent in chronic AVB than in SR, thus further unmasking the presence of intrinsic repolarization disturbances. In addition, chronic-AVB cells of both ventricles show an increased susceptibility to EAD generation. Although the present study does not elucidate the ionic basis of these action-potential differences or the genesis of ventricular ectopic beats, it appears that phenotypic alterations of repolarization are involved in the ventricular arrhythmias and sudden cardiac death of dogs with chronic AVB.

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Downregulation of Delayed Rectifier K⁺ Current in Dogs with Chronic Complete Atrioventricular Block and Acquired Torsades de Pointes

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65

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Abstract

Background: Acquired QT prolongation enhances the susceptibility to torsades de pointes (TdP). Clinical and experimental studies indicate ventricular action-potential prolongation, increased regional dispersion of repolarization and early afterdepolarizations as underlying factors. We examined whether K^+ -current alterations contribute to these proarrhythmic responses in an animal model of TdP: the dog with chronic complete atrioventricular block (AVB).

Methods and Results: Whole-cell K^+ currents I_{TO1} , I_{K1} , I_{Kr} , and I_{Ks} were recorded in left (LV) and right ventricular (RV) midmyocardial cells from dogs with 9 ± 1 weeks of AVB and controls with sinus rhythm. I_{TO1} density and kinetics, and I_{K1} outward current were not different between chronic AVB and control. I_{Kr} , measured as the almokalant-sensitive tail current on repolarization to -50 mV, had a similar voltage dependence of activation and time course of deactivation in chronic AVB and control. I_{Kr} density was similar in LV myocytes, but smaller in RV myocytes ($\sim 45\%$) of chronic AVB versus control. For I_{Ks} , measured as the tail current on repolarization to -25 mV in $0 [K^+]_o$ and the presence of almokalant, voltage dependence of activation and time course of deactivation were similar in chronic AVB and control. However, I_{Ks} densities of LV ($\sim 50\%$) and RV cells ($\sim 55\%$) were significantly lower in chronic AVB than control.

Conclusions: Significant downregulation of delayed rectifier K^+ current occurs in both ventricles of the dog with chronic AVB. The low functional expression of I_{Ks} predisposes to acquired TdP in vivo, and suggests that this animal model develops intrinsic repolarization defects as found in the human long-QT syndrome.

Introduction

Acquired QT prolongation is a common electrocardiographic finding in heart disease, but can also be present as a primary derangement in the absence of structural abnormalities or drugs.¹ It is regarded as one of the risk factors of ventricular arrhythmias and sudden cardiac death.¹ Clinical and experimental studies on the acquired long-QT syndromes indicate an enhanced susceptibility to polymorphic ventricular tachycardias, notably torsades de pointes (TdP).^{2,3} Unlike for the congenital long-QT syndromes, in which distinctive defects of ion-channel function in myocardial cells are found to be responsible for delayed repolarization,⁴ the ionic basis of acquired QT prolongation, not related to drugs, is poorly understood.

In recent years we have used the dog with chronic complete atrioventricular block (AVB) as a model for the study of TdP.⁵ QT intervals and ventricular endocardial monophasic action-potential durations (APDs) are much longer than expected on the basis of the bradycardia alone in these animals and point towards a disturbed ventricular repolarization,⁶ corresponding with clinical findings on acquired AVB.^{7,8} Anesthetized dogs show an enhanced susceptibility to acquired TdP after several weeks of AVB duration, which is associated with the development of increased interventricular dispersion of repolarization and early afterdepolarizations.⁶ Electrical remodeling is accompanied by the development of biventricular hypertrophy.^{6,9} Approximately 15% of the dog population dies suddenly, often during circumstances of excitement (eg, during feeding or ambulation). Transmembrane action-potential recordings in isolated myocytes indicate that the prolonged ventricular repolarization of chronic AVB is an intrinsic abnormality, which is amplified by class III antiarrhythmic drugs.⁹ In addition, action-potential prolongation is more pronounced in left (LV) than right ventricular (RV) myocytes, supporting the in-vivo finding of increased regional dispersion of repolarization.

In our search for the ionic mechanisms of electrical remodeling and proarrhythmia in dogs with chronic AVB, we investigated the possible contribution of K^+ -current alterations to ventricular action-potential prolongation and increased regional dispersion of repolarization. To this end, we measured whole-cell K^+ currents in midmyocardial cells of animals with documented TdP and directly compared LV and RV myocytes in the same hearts.

Methods

Animal handling was in accordance with the 'Dutch Law on Animal Experimentation' and the 'European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EU)'. The experiments were approved by the 'Committee for Experiments on Animals' of our university.

In-Vivo Studies

Twenty four adult mongrel dogs of either sex and weighing between 22 and 37 kg were used for the experiments. For a complete description of the creation of AVB ($n_{\text{dogs}}=15$) and perioperative care we refer to a previous publication.⁶ To test the induction of TdP, the class III agent almokalant (0.12 mg/kg/10 minutes IV) was used, either alone (spontaneous TdP) or in combination with programmed electrical stimulation.¹⁰ TdP was defined as a polymorphic ventricular tachycardia consisting of ≥ 5 beats twisting around the baseline in a setting of prolonged QT(U) duration. These experiments were performed during anesthesia =1 week prior to sacrifice of the dogs for cell isolation.

Cellular Experiments

Anesthetized chronic-AVB (9 ± 1 weeks) and control dogs (with sinus rhythm; $n_{\text{dogs}}=9$) received heparin IV upon thoracotomy. The hearts were quickly excised and washed in cold cardioplegic solution. Heart weights of chronic-AVB dogs were significantly larger than those of control dogs: 306 ± 12 versus 220 ± 8 g, respectively ($P < 0.05$). When corrected for body weight, this difference remained significant: 11.6 ± 0.3 versus 8.6 ± 0.3 g/kg ($P < 0.05$).

To isolate single LV and RV midmyocardial cells simultaneously, the left-anterior-descending and right coronary artery were cannulated. The isolation procedure was the same as recently described by our group.¹¹ Myocytes were stored at room temperature in standard-buffer solution. Whole-cell currents were measured using patch pipettes (borosilicate glass) with resistances of 1.0 to 3.0 M Ω when filled with internal solution. Experiments were performed at $37 \pm 0.5^\circ\text{C}$. Cell capacitance, measured by hyperpolarizing steps from a holding potential of -60 mV, was similar in the four cell groups. In the LV myocytes, average values were 216 ± 9 pF ($n_{\text{Chronic AVB}}=35$) versus 227 ± 11 pF ($n_{\text{Control}}=27$; $P=\text{NS}$). In RV myocytes, capacitances were 221 ± 12 pF ($n_{\text{Chronic AVB}}=28$) versus 228 ± 11 pF ($n_{\text{Control}}=29$; $P=\text{NS}$).

For the measurements of I_{TO1} , we used a holding potential of -70 mV. Na^+ current was inactivated by a 10-ms prepulse to -40 mV. L-type Ca^{2+} current was inhibited with nifedipine (5 $\mu\text{mol/L}$). The activation protocol consisted of 300-ms test pulses (V_{test}) to -20 up to +70 mV with 10-mV increments and 10-s intervals. I_{TO1} amplitudes were measured as peak amplitudes minus steady-state values at the end of depolarizations. For the measurements of I_{K1} , I_{Kr} , and I_{Ks} , the holding potential was set at -50 mV. I_{K1} activation was tested during 200-ms pulses from -20 to -140 mV with 10-mV decrements every 3 s. Sustained outward currents were recorded during prolonged (3-s) V_{test} of -20 to +70 mV with 10-mV increments and 20-s intervals. I_{Kr} and I_{Ks} were measured as the peak tail currents upon repolarization. For I_{Kr} , we used the tail current sensitive to 2 $\mu\text{mol/L}$ almokalant (specific I_{Kr} blocker) upon repolarization to the holding potential of -50 mV. For I_{Ks} , almokalant-resistant tail currents were used.

The standard-buffer solution used for the experiments was of the following composition (mmol/L): NaCl 145, KCl 4.0, CaCl_2 1.8, MgCl_2 1.0, NaH_2PO_4 1.0,

glucose 11, HEPES 10, pH 7.4 with NaOH at 37°C. The patch-pipette solution contained (in mmol/L): potassium aspartate 125, KCl 20, $MgCl_2$ 1.0, MgATP 5, HEPES 5, EGTA 10, pH 7.2 with KOH.

The data are expressed as mean $\pm$ SEM. Intergroup comparisons were made with the Student's *t* test for unpaired and paired data groups, after testing for the normality of distribution. Differences were considered significant if $P < 0.05$.

Results

Acquired QT Prolongation and Susceptibility to TdP in the Study Group

In-vivo electrophysiological recordings during sinus rhythm, and during acute and chronic AVB revealed significant QT prolongations in all chronic-AVB dogs. An example of these three states in the same animal is shown in Figure 1. On average, the QT time (measured in ECG lead II) increased from 241 ± 9 ms at sinus rhythm to 316 ± 14 ms upon creation of AVB ($P < 0.05$), and to 374 ± 15 ms in the chronic phase at 9 ± 1 weeks thereafter ($P < 0.05$ versus acute AVB). Corresponding RR intervals measured 488 ± 24 ms, 1479 ± 142 ms ($P < 0.05$), and 1381 ± 80 ms, respectively ($P = NS$ versus acute AVB). From acute to chronic AVB, QRS widths did not change significantly (see Figure 1). However, broad-based T waves developed early after the QRS complex. In 13 of the 15 animals with chronic AVB, LV and RV endocardial monophasic action potentials were recorded at the idioventricular cycle length. In all 13 the LV APD was longer than the RV APD: on average 419 ± 25 ms versus 354 ± 19 ms ($P < 0.05$), yielding an interventricular difference of 65 ± 12 ms (in line with previous results^{6,10}). The susceptibility to TdP was tested in 7 animals using alkemokant. In 6 of these dogs spontaneous TdP occurred, in the other dog TdP could not be induced.

In the sinus-rhythm control group used for comparisons in the cellular investigations, the RR interval was 421 ± 22 ms with a QT time of 231 ± 6 ms (both $P = NS$ versus preoperative sinus rhythm in the chronic-AVB group).

I_{TO1} Is Not Altered in Chronic AVB

The activation of I_{TO1} was tested during steps of -40 to $+70$ mV. In all four cell types the current activated at $V_{test} \geq -20$ mV (Figure 2). There was no difference in I_{TO1} density between chronic AVB and control in both ventricles. The difference in I_{TO1} between LV and RV, recently described for normal canine hearts,¹¹ was maintained in chronic AVB. I_{TO1} was nearly completely inhibited by 5 mmol/L 4-aminopyridine in all four cell types. I_{TO1} inactivation during the 300-ms V_{test} was best fitted with a single exponential function yielding similar time constants (range 7 to 14 ms) in chronic AVB and control. As illustrated in Figures 3A and 3B, voltage dependence of steady-state inactivation and time-dependent recovery from inactivation were not different between the cell types.

Figure 1 (left page). – Acquired QT prolongation in a dog with AVB.

Shown are the ECG leads II and AVF, and simultaneous monophasic-action-potential recordings from the LV (LV MAP) and RV endocardium (RV MAP) at a paper speed of 25 mm/s. During sinus rhythm (1 CONTROL) the cycle length is 585 ms and the QT time 280 ms. LV and RV MAP duration measure 230 and 220 ms, respectively. When AV-nodal conduction is blocked with formaldehyde during the same session (2 AAVB), bradycardia ensues with an idioventricular cycle length of 1570 ms. At this moment there is a minimal QT prolongation to 285 ms, while LV and RV MAP durations are 310 and 290 ms. In the right panel (3 CAVB), the effect of 6 weeks AVB on electrophysiological parameters is obvious: at a similarly slow idioventricular rhythm (cycle length 1460 ms) the QT time has increased significantly to 580 ms with MAP prolongation to 540 and 430 ms in LV and RV, leading to an interventricular difference of 110 ms. Note the broad-based, high-amplitude T waves in II and AVF. Note also that the QRS duration is unaltered in CAVB, compared to CONTROL and AAVB. TdP occurred spontaneously in this animal during administration of almokalant.

Properties of I_{K1}

Figure 4 shows typical recordings of I_{K1} in chronic-AVB and control myocytes, as well as current density-voltage relations at the end of V_{test} of -140 to -20 mV in both LV and RV. The current showed rapid activation and was fully inhibited in K^+ -free superfusate (0 $[K^+]_O$; not shown). I_{K1} outward currents (initial peak values as well as steady-state levels) at V_{test} positive to the K^+ reversal potential were similar in chronic AVB and control. Only at very hyperpolarizing pulses in RV cells, current densities were less negative in chronic AVB. There were no interventricular differences between chronic AVB and control.

Downregulation of I_{Ks} and I_{Kr}

To dissect the two components of the delayed rectifier K^+ current at baseline, we applied a protocol in which, after a depolarization to +30 mV, repolarization was divided in two steps: to 0 mV for 4.5 s and then back to -50 mV (Figure 5).¹² The hypothesis that tail currents at the first repolarization step were largely composed of deactivating I_{Ks} and largely devoid of I_{Kr} was confirmed by the observation that they were insensitive to almokalant (Figure 5, first arrow in both panels). In contrast, tail currents at the second repolarization step decreased significantly on almokalant administration, indicating predominately the presence of I_{Kr} (Figure 5, second arrow in both panels). From these data it appeared that I_{Ks} (tail currents at first repolarization step) was smaller in chronic AVB than control myocytes: for LV, 0.19 ± 0.02 versus 0.40 ± 0.09 pA/pF, and for RV 0.21 ± 0.03 versus 0.51 ± 0.08 pA/pF, respectively (both $P < 0.05$). For the same comparison, I_{Kr} (tail currents at second repolarization step) was similar in LV, 0.34 ± 0.03 versus 0.42 ± 0.05 pA/pF ($P = \text{NS}$), but smaller in RV myocytes, 0.34 ± 0.02 versus 0.48 ± 0.02 pA/pF in chronic AVB versus control, respectively ($P < 0.05$). To elaborate further on the contribution of I_{Ks} to total delayed rectifier K^+ current, we performed envelope-of-tails tests at baseline and in the presence of almokalant. These experiments revealed that I_{Ks} made up a significant portion of the total current, even for depolarizations as short as 300 ms. However, contributions were much smaller in chronic-AVB than control cells (data not shown).

Figure 2. – I_{TO1} activation in chronic AVB.
Shown are families of current traces during V_{test} of -20 to +70 mV (interval 10 s) in two RV myocytes (capacitances: 264 and 247 pF in Chronic AVB and Control, respectively). Left horizontal bars indicate 0-pA level. Lower panels, current-density-voltage relations for LV ($n_{Chronic\ AVB}=17$, $n_{Control}=10$) and RV ($n_{Chronic\ AVB}=16$, $n_{Control}=12$). Inset on the right, voltage-clamp protocol.

Figure 3. – **A**, Voltage-dependence of steady-state inactivation of I_{T01} . Upper left panel, family of traces in a chronic-AVB LV cell. Right inset, voltage-clamp protocol. Lower panels, fractions of I_{T01} relative to amplitude at V_{test} of +60 mV for a V_{cond} of -100 mV in LV ($n_{Chronic\ AVB}=8$, $n_{Control}=9$) and RV ($n_{Chronic\ AVB}=4$, $n_{Control}=6$). Curves are Boltzmann fits to the data. Half points and slope factors are similar in the four groups. **B**, Time-dependent recovery from inactivation. Upper left panel, original traces with interpulse delays (Δt) of 5 to 50 ms (5-ms increments), 60 to 180 ms (20-ms increments), and 2000 ms in a chronic-AVB LV myocyte. Right inset, voltage-clamp protocol indicates prepulse to +60 mV followed by V_{test} to +60 mV at increasing Δt . Lower panels, fractions of I_{T01} relative to amplitude in prepulse as a function of Δt in LV ($n_{Chronic\ AVB}=6$, $n_{Control}=8$) and RV ($n_{Chronic\ AVB}=3$, $n_{Control}=7$). Left horizontal bars in original traces indicate 0-pA levels.

I_{K1}

Chronic AVB

Control

Left ventricle

Right ventricle

Figure 4. I_{K1} in chronic AVB.

Examples are from LV myocytes and show currents during V_{test} of -70 to -140 mV (interval 3 s). Lower panels, current density-voltage relations for I_{K1} steady-state activation in LV ($n_{Chronic\ AVB}=8$; $n_{Control}=5$) and RV ($n_{Chronic\ AVB}=10$; $n_{Control}=9$). In RV, * indicates significant difference ($P<0.05$).

Figure 5. – Activation and deactivation of delayed rectifier K^+ current at baseline and during almokalant ($2 \mu\text{mol/L}$) treatment in chronic AVB and control.

Shown is the activation in response to a 3-s depolarization to $+30 \text{ mV}$, followed by deactivation phases at 0 mV for 4.5 s and at -50 mV in two LV cells (capacitances: Chronic AVB, 292 pF and Control, 299 pF). In both panels, the upper of the two current traces is the baseline recording, whereas the lower trace is the current during almokalant a few intervals later (interval 20 s). Tail-current amplitudes at the first repolarization step (first arrow) are unaltered during almokalant, whereas tail currents at the second repolarization step (second arrow) are decreased significantly. In the comparison Chronic AVB versus Control, tail-current amplitudes at the first repolarization step are markedly lower in Chronic AVB, indicating a lower I_{Ks} . Similar results were obtained for LV in $n_{\text{Chronic AVB}}=8$ and $n_{\text{Control}}=11$, and for RV in $n_{\text{Chronic AVB}}=11$ and $n_{\text{Control}}=16$.

Voltage-dependent activation of I_{Ks} was evaluated from tail currents on repolarization to -25 mV in $0 [\text{K}^+]_O$ in the presence of almokalant. Examples of families of current traces and pooled data are given in Figure 6. There was no saturation of tail-current amplitudes. Voltage dependence of I_{Ks} activation was similar in the four cell types. However, I_{Ks} density was significantly smaller in chronic AVB than in control. Overall, tail currents were reduced by 50% in LV and by 55% in RV myocytes (Figure 6). Whereas interventricular differences of I_{Ks} exist in normal canine hearts (Figure 6),¹¹ due to the small current amplitudes in chronic AVB no differences could be discerned. Voltage dependence of I_{Ks} deactivation was not different between chronic AVB and control. Likewise, the time course of deactivation proved similarly fast in all four cell types.

Figure 6. $-I_{Ks}$ in chronic AVB.

Shown are currents for depolarizations to -20 to +60 mV (20-mV increments; 20-s intervals) followed by repolarizations to -25 mV in 0 [K⁺]_o superfusate with 2 μ mol/L almokalant in two LV myocytes (capacitances: 213 and 251 pF in Chronic AVB and Control, respectively). Left horizontal bars indicate 0-pA level. Lower panels, activation for I_{Ks} tail currents on repolarization to -25 mV in LV ($n_{\text{Chronic AVB}}=12$, $n_{\text{Control}}=5$) and RV ($n_{\text{Chronic AVB}}=9$, $n_{\text{Control}}=9$). In both ventricles, * indicates significant difference ($P < 0.05$). Inset, voltage-clamp protocol.

Figure 7. – I_{Kr} in chronic AVB.

Upper panels, I_{Kr} is evaluated as the almokalant-sensitive difference current after digital subtraction of the baseline current and the current remaining during subsequent treatment with 2 $\mu\text{mol/L}$ almokalant. Tail currents are measured on repolarization to -50 mV (arrows) and are plotted as a function of V_{cond} in the lower panels. In LV, $n_{\text{Chronic AVB}}=18$ and $n_{\text{Control}}=8$. In RV, $n_{\text{Chronic AVB}}=14$ and $n_{\text{Control}}=13$; * indicates significant difference ($P<0.05$). Left horizontal bars, 0-pA level. Insets, voltage-clamp protocols.

I_{Kr} was quantified as the almokalant-sensitive tail-current portion measured by digital subtraction on single-step repolarizations to -50 mV in 4.0 mmol/L $[K^+]_O$ (Figure 7). Activation occurred after depolarizations ≥ -10 mV, and showed saturation at conditioning voltages (V_{cond}) $> +20$ mV. Boltzmann fits to the data revealed half points of 0.3 ± 1.1 mV and -1.1 ± 3.1 mV in LV, and of -2.5 ± 2.1 mV and 1.1 ± 1.7 mV in RV for chronic AVB and control, respectively (both $P=NS$). In the same order, corresponding slope factors were 5.8 ± 1.0 mV and 5.7 ± 2.8 mV in LV, and 5.5 ± 1.7 mV and 5.5 ± 1.5 mV in RV (both $P=NS$). I_{Kr} density was similar in LV myocytes, but smaller in RV myocytes of chronic AVB versus control (reduction of 45%; Figure 7). There were no interventricular differences. In both ventricles, voltage dependence and time course of I_{Kr} deactivation were similar for chronic AVB and control.

Discussion

We have demonstrated that acquired QT prolongation in dogs with chronic AVB and documented TdP is associated with significant reductions of I_{Ks} in the LV and RV. In the RV of these animals, I_{Kr} is also downregulated. Of the other K^+ currents, I_{TO1} and I_{K1} outward current are unaltered.

Reproducible Induction of TdP in Chronic AVB

The dog with chronic AVB is a very suitable large-animal model for the study of TdP. In the majority of anesthetized animals the arrhythmia can be evoked in a reproducible fashion with class III antiarrhythmic agents alone or combined with programmed electrical stimulation.⁵ In-vivo experiments indicate the critical importance of regional dispersion of repolarization, early afterdepolarizations and multiple ectopic beats for the initiation of TdP.^{10,13} Interventricular dispersion during arrhythmogenesis probably reflects the existence of significant repolarization gradients in closely adjacent areas, possibly the septum.¹⁴ Our most recent results demonstrate that electrical remodeling of the myocardium is the substrate for the enhanced susceptibility to TdP.^{6,9} The in-vivo part of the present study was not designed to reinvestigate the criteria for TdP, but confirmed that the dogs used for cellular experiments were a representative population with significant QT prolongation and a low threshold for TdP.

Contribution of Reduced I_{Ks} and I_{Kr} to Action-Potential Prolongation

In a previous study, at serial testing in vivo, it was found that chronic (versus acute) AVB leads to increases of endocardial monophasic APDs of approximately +30% in the LV, and +20% in the RV.⁶ Based on our recent microelectrode study,⁹ the relative increase of the APD in chronic-AVB myocytes is 10 to 30% in the LV (depending on the pacing cycle length), and maximally 10% in the RV under baseline conditions. In these cells the class III agents almokalant and *d*-sotalol cause marked action-potential prolongation and the occurrence of early afterdepolarizations, which

uncovers the importance of I_{Kr} for ventricular repolarization in chronic AVB. Whether a 50-55% reduction of I_{Ks} can account for the action-potential characteristics in LV and RV can only be answered indirectly. In a recent study on canine LV midmyocardial tissue, the I_{Ks} blocker chromanol 293B at 30 $\mu\text{mol/L}$ increases the APD from 284 ± 13 to 357 ± 25 ms at a pacing cycle length of 2000 ms (+25%).¹⁵ This concentration reduces (guinea-pig) I_{Ks} by approximately 80%.^{16,17} In a theoretical model of the normal human ventricular action potential,¹⁸ adapted from the Luo-Rudy model,¹⁹ 50% inhibition of I_{Ks} increases the APD from 374 to approximately 425 ms (+15%; their Figure 12). Thus, at rough approximation, the 50-55% decrease of I_{Ks} observed in our present study could account for a 10-30% action-potential prolongation, at least in the LV. The RV exhibits only minor action-potential alterations under baseline conditions, despite its additional downregulation of I_{Kr} (-45%). To explain why the alterations are less pronounced in the RV than LV we have to consider that the basic determinants of repolarization are different between the two ventricles. Recently, we have described that this is indeed the case in the normal canine heart: I_{TO1} and I_{Ks} are much larger in the RV, indicating a larger repolarization reserve.¹¹ In chronic AVB the difference in I_{TO1} is maintained, and it is likely that also other membrane currents and/or their remodeling are responsible for the amplification of interventricular action-potential inhomogeneities. We are currently investigating two candidates: the L-type Ca^{2+} current and $\text{Na}^{+}\text{-Ca}^{2+}$ exchange.²⁰

Ionic Remodeling in Chronic AVB

Cardiac function of dogs with AVB of 9-weeks' duration is unimpaired.^{6,20} Most animals have an enhanced contractile performance at the imposed bradycardia and lack signs of heart failure. At correspondingly slow pacing rates in single myocytes, Ca^{2+} release from the sarcoplasmic reticulum and contractions are larger than in control cells.²⁰ Significant growth responses (merely of cell length) are observed in both RV (+23%) and LV (+13%) myocytes,⁹ while autopsy findings reveal increased RV and LV tissue weights.⁶ These data strongly support the contention that dogs with long-standing (weeks to months) AVB have a compensated form of biventricular hypertrophy.^{21,22}

In many other animal models and in humans with cardiac hypertrophy or failure, downregulation of K^{+} currents has been implicated in (inhomogeneous) ventricular action-potential prolongation,²³ and the increased risk of ventricular arrhythmias and sudden cardiac death through increased dispersion of repolarization.²⁴ Reduction of I_{TO1} is probably the most often observed in the spectrum of early compensated hypertrophy to terminal heart failure,²⁵ and has been linked to action-potential prolongation.^{26,27} However, it has been questioned whether downregulation of this current alone can cause increased APDs in large mammals including humans.¹⁸ Reduction of I_{TO1} is clearly absent in dogs with chronic AVB, which corresponds to the finding of marked notch amplitudes in midmyocardial action potentials (RV > LV).⁹ There have been only a few reports on the downregulation of delayed rectifier K^{+} current in cardiac

hypertrophy induced by pressure overload in cats.^{28,29} In these studies, a role for the reduced total current in the increase of APDs was inferred, but the investigators did not discriminate between I_{Kr} and I_{Ks} . Our data do make this distinction for the hypertrophied cells of dogs with chronic AVB and indicate that changes of these relatively small currents can have major impact on the course of repolarization, as noted before.³⁰

The Dog with Chronic AVB: A Model of the Human Long-QT Syndrome?

The combined findings of an enhanced susceptibility to acquired TdP, the (supposed) adrenergic dependence of spontaneous episodes of TdP, the typical T-wave patterns during prolonged QT intervals, and the reduction of I_{Ks} in this animal model resemble the clinical characteristics of the LQT1 form of the human congenital long-QT syndrome. Distinctive mutations in three ion channels (the slow and rapid delayed K^+ channels, and the Na^+ channel) have been identified in this syndrome, which could be linked to inherited arrhythmias.⁴ The LQT1 form, with a reduced I_{Ks} , is the most often encountered in patients and carries the highest risk of cardiac events (syncope, aborted cardiac arrest, or sudden death)³¹ due to the generation of TdP. Experimental electrophysiological studies on the generation of TdP in the long-QT syndrome have often used models in which the phenotypes of reduced I_{Ks} (LQT1 or LQT5)¹⁵ or I_{Kr} (LQT2),^{32,33} or of prolonged late Na^+ current (LQT3)³²⁻³⁴ were mimicked by pharmacological interventions. The dog with chronic AVB may prove to be a valuable addition to these surrogates and could be favorable in experimental setups requiring a longitudinal design. Although the current reductions in this model are not the consequence of genetic mutations as in the congenital long-QT syndrome, the intrinsic nature of these functional alterations may constitute another advantage over the artificial inhibition with pharmacological agents.

Conclusions

Significant downregulation of delayed rectifier K^+ current contributes to the repolarization abnormalities in the LV and RV of dogs with chronic AVB. The low functional expressions of I_{Ks} and I_{Kr} , in combination with the maintained interventricular difference in I_{TO1} and possibly other membrane currents, are responsible for the amplification of interventricular action-potential inhomogeneities at control. The resultant increased regional dispersion of repolarization constitutes the substrate for an enhanced susceptibility to acquired TdP. Thus, this animal model carries intrinsic repolarization defects and in-vivo proarrhythmia as in the human congenital long-QT syndrome.

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Functional Upregulation of $\text{Na}^+\text{-Ca}^{2+}$ Exchange in Cardiac Hypertrophy May Contribute to Contractile Adaptation, But Also to Arrhythmogenesis

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Abstract

Background: Triggered arrhythmias are a major cause of sudden death in patients with heart failure and hypertrophy. The dog with chronic complete atrioventricular block (AVB) has proarrhythmia and biventricular hypertrophy, and is a useful model to study cellular mechanisms of arrhythmias. We investigated whether contractile adaptation to chronic AVB and changes in Ca^{2+} homeostasis might contribute to proarrhythmia.

Methods and Results: Contractile function in vivo was enhanced in chronic AVB, in particular at the (slow) idioventricular heart rate. In enzymatically isolated single cells, shortening and Ca^{2+} release from the sarcoplasmic reticulum (SR) were likewise enhanced at low stimulation frequencies. Ca^{2+} influx through L-type Ca^{2+} channels was unchanged, but Ca^{2+} influx via the $\text{Na}^+\text{-Ca}^{2+}$ exchanger was significantly larger, and contributed to Ca^{2+} loading of the SR. Inward $\text{Na}^+\text{-Ca}^{2+}$ -exchange currents were larger in left ventricular cells from chronic AVB. In right ventricular cells of chronic AVB, changes in Ca^{2+} fluxes were less pronounced.

Conclusions: Enhanced $\text{Na}^+\text{-Ca}^{2+}$ -exchange activity may improve contractile adaptation to chronic AVB, but may at the same time contribute to proarrhythmia, by (1) providing more inward current at the time of Ca^{2+} release, leading to increased dispersion of repolarization at slow heart rates; (2) increasing the propensity to Ca^{2+} overload; and (3) enhancing (arrhythmogenic) currents during spontaneous Ca^{2+} release.

Introduction

Enhanced susceptibility to arrhythmias in hypertrophy and heart failure is due to structural and functional, including electrical, remodeling; disturbances in repolarization, with QT prolongation and enhanced dispersion of repolarization, provide the substrate for initiating and sustaining ventricular arrhythmias.¹⁻³ The underlying cellular changes have been a major topic of investigations in recent years. In myocytes isolated from human failing hearts, a regionally variable reduction in the transient outward K^+ current, I_{to} , was identified,^{4,5} and similar changes were observed in animal models of heart failure and hypertrophy (eg, References 6 and 7). The role of a reduction of I_{to} in action-potential prolongation, however, is not yet resolved, and other membrane currents may be of major importance. Changes in other K^+ currents and in Ca^{2+} currents have been identified in animal models of hypertrophy (reviewed in Reference 8). Another ionic current possibly involved in action-potential prolongation is the $\text{Na}^+\text{-Ca}^{2+}$ exchanger. This Ca^{2+} transporter exchanges 1 Ca^{2+} for 3 Na^+ ions, and generates an inward current during Ca^{2+} efflux; Ca^{2+} influx via the $\text{Na}^+\text{-Ca}^{2+}$ exchanger (reverse mode) will enhance Ca^{2+} loading of the cell.⁹ Its activity as a Ca^{2+} -extrusion pathway, and thus the amplitude of the inward current, is closely linked to the amplitude and time course of the $[\text{Ca}^{2+}]_i$ transient and contributes to the plateau of the action potential.^{10,11} Prolongation of the $[\text{Ca}^{2+}]_i$ transient due to a decrease in sarcoplasmic reticulum (SR) Ca^{2+} pumps, may lead to larger exchanger currents in heart failure,¹² and contribute to action-potential prolongation in this condition.¹³ Studies of human failing hearts have found an increase in the level of expression of the $\text{Na}^+\text{-Ca}^{2+}$ exchanger.^{14,15} From studies of mice with overexpression of the $\text{Na}^+\text{-Ca}^{2+}$ exchanger, it has become clear that an increase in the level of $\text{Na}^+\text{-Ca}^{2+}$ -exchanger proteins will affect Ca^{2+} homeostasis and membrane currents.¹⁶⁻¹⁸ Through its effects on membrane currents and $[\text{Ca}^{2+}]_i$, changes in $\text{Na}^+\text{-Ca}^{2+}$ exchange during ventricular hypertrophy and remodeling can shape the substrate as well as the trigger for arrhythmias. However, the link between arrhythmogenesis and changes in Ca^{2+} homeostasis, in particular the $\text{Na}^+\text{-Ca}^{2+}$ exchanger, is largely unexplored.

The dog with chronic complete atrioventricular block (AVB) has proven to be an excellent model for the study of triggered arrhythmias, linked to a prolongation and increased dispersion of repolarization, and to the occurrence of afterdepolarizations.¹⁹ The proarrhythmia typically develops in parallel with biventricular hypertrophy and remodeling. Hypertrophy and action-potential prolongation are intrinsic characteristics of the myocytes.²⁰ A decrease in delayed rectifier K^+ current was implicated in the action-potential prolongation,²¹ and confirms the presence of ionic remodeling. Therefore this model offers the unique opportunity to further characterize the cellular substrate of triggered arrhythmias in the setting of remodeling. In the present study we will explore alterations in cellular Ca^{2+} homeostasis, in particular changes in Ca^{2+} release from the SR and in Ca^{2+} transport by the (electrogenic) $\text{Na}^+\text{-Ca}^{2+}$ exchanger, which may contribute to proarrhythmia.

Methods

In-Vivo Studies

The procedures for creating AVB and for anesthesia were as described before.²² All experiments were approved by the university 'Committee for Experiments on Animals' (Leuven, Maastricht) and performed in accordance with the European Directive for the Protection of Vertebrate Animals and other Scientific Purposes. In a total of 10 anesthetized mongrel dogs of either sex left ventricular (LV) pressure was recorded during LV epicardial pacing with a single micromanometer pressure transducer catheter (Sentron, Roden, Netherlands), as previously described.²²

Cell Isolation

A total of 18 dogs were studied after 8 ± 1 weeks of AVB. A group of 15 dogs in sinus rhythm, with comparable body weight (BW) and age, served as control. The procedure for cell isolation has been described in detail before.^{20,23} Briefly, the heart was removed under deep anesthesia. Heart weight (HW) was immediately determined, and for all chronic-AVB dogs, hypertrophy was confirmed by a HW/BW of 11.4 ± 0.4 g/kg versus 8.0 ± 0.2 g/kg for control dogs ($P < 0.001$). Both the left-anterior-descending coronary artery and the right coronary artery were cannulated and the tissue perfused with enzymes, to obtain LV and right ventricular (RV) cells from the same hearts.²³ After removal of the epicardial layer, midmyocardial cells were sampled with a pasteur pipette, avoiding contamination by the endocardium. Isolated myocytes were stored in a normal Tyrode's solution (see below) at room temperature.

Recording of Cell Shortening, Membrane Currents, and $[\text{Ca}^{2+}]_i$

All experiments were performed at $36\text{--}37^\circ\text{C}$. Cells were studied on an inverted microscope (Nikon Diaphot). Shortening of cells impaled with high-resistance microelectrodes was measured with a video-edge detector (Crescent Electronics).

Membrane currents were recorded in the whole-cell, ruptured patch-clamp mode with an Axopatch 1D amplifier, filtered at 1 kHz and sampled and digitized at 4 kHz (Fastlb45, Indec Systems). Cell capacitance, determined during short hyperpolarizing pulses from -60 mV to -70 mV, was 155 ± 6 pF ($n=48$) in chronic-AVB LV myocytes versus 135 ± 9 pF ($n=39$) in control ($P < 0.05$), and capacitance of RV myocytes was 150 ± 11 pF ($n=30$) in chronic AVB versus 109 ± 6 pF ($n=39$) in control ($P < 0.05$).

$[\text{Ca}^{2+}]_i$ was monitored with fluo-3 ($50 \mu\text{mol/L}$) or a combination of fluo-3 ($30 \mu\text{mol/L}$) and fura-red ($70 \mu\text{mol/L}$). The setup and calibration were as described before.²⁴ With fluo-3 alone, fluorescence values were background-corrected and normalized for baseline fluorescence (pseudo-ratio); $[\text{Ca}^{2+}]_i$ was calculated assuming a K_D of 400 nM and a resting value of 100 nM.²⁵ Previous studies have documented the use of fluo-3 and pseudo-ratios in comparing $[\text{Ca}^{2+}]_i$ transients from normal cells to transients from hypertrophied cells.²⁶ Values obtained with this latter approach correlated well with the calibrated values obtained with the dual-dye method.

Pooled data are shown as mean $\pm$ SEM. Comparisons between groups were made with Student's unpaired t test, or a χ^2 test where appropriate. P values <0.05 were considered significant.

Solutions and Experimental Protocols

For measurement of $[\text{Ca}^{2+}]_i$, the pipette solution contained (in mmol/L): K-aspartate 120, KCl 20, K-HEPES 10, MgATP 5, MgCl_2 0.5, NaCl 10 or 20, fluo-3 0.05 (or fluo-3 0.03 and fura-red 0.07); pH 7.20. In some experiments, K-aspartate and KCl were replaced with Cs-aspartate and CsCl respectively. The external solution contained (in mmol/L): NaCl 130, KCl 5.4, Na-HEPES 11.8, MgCl_2 0.5, CaCl_2 1.8, glucose 6; pH 7.35.

For measurement of the L-type Ca^{2+} current (I_{CaL}), the pipette solution contained (in mmol/L): NMDGCl 110, TEACl 20, HEPES 10, MgCl_2 0.5, MgATP 5, Cs-EGTA 10; pH 7.20 with TEOH. The external solution contained (in mmol/L): NMDGCl 130, HEPES 10, glucose 10, MgCl_2 0.5, CaCl_2 1.8; pH 7.40 with TEOH.

To block L-type Ca^{2+} channels we used 20 $\mu\text{mol/L}$ nisoldipine (Bayer) or 20 $\mu\text{mol/L}$ nifedipine (Sigma), both prepared as a 20 mmol/L stock in DMSO. Ca^{2+} release from the SR was disabled with 10 $\mu\text{mol/L}$ ryanodine (Sigma) prepared as a 10 mmol/L stock in water. In the presence of nisoldipine and ryanodine, NiCl_2 (2.5 mmol/L) was used to block $\text{Na}^+\text{-Ca}^{2+}$ exchange. To minimize effects of Ni^{2+} on time-dependent K^+ currents, we measured Ni^{2+} -sensitive currents at 30 ms into the depolarizing step. Current densities measured with all K^+ replaced with Cs^+ , were not different from those with K^+ (1.25 ± 0.62 pA/pF at +70 mV versus 1.23 ± 0.65 pA/pF, in each group 3 cells from the same heart), as we reported previously.²⁴

Results

Contractile Function in Vivo and In Vitro

Peak LV dP/dt was measured during LV epicardial pacing (Figure 1A). After the creation of AVB, an increase in pacing frequency resulted in an increase in LV dP/dt. At this time, LV-end diastolic pressure (LV EDP) was 15 ± 2 mm Hg at the lowest frequency (versus 11 ± 1 mm Hg at sinus rhythm), and did not change significantly with pacing frequency. After 6 weeks of AVB, LV dP/dt at the lowest frequency was twice as large as at the time of acute AVB ($P<0.01$). LV dP/dt decreased with increasing frequency, but was still significantly larger than at acute AVB. LV EDP after 6 weeks of AVB was 11 ± 2 mm Hg at the low frequency and did not change significantly at the higher frequency.

In single LV cells from control dogs and impaired with high-resistance microelectrodes, increasing the frequency of stimulation tended to increase the extent of shortening ($P=\text{NS}$, Figure 1B). In cells from chronic-AVB dogs, the extent of shortening

Figure 1. – Contractile function in chronic AVB.

A, In-vivo function was evaluated from the LV dP/dt. Control values (open circles) were obtained immediately after the creation of AVB ($n_{\text{dogs}}=6$). Dogs were tested again after 6 weeks of AVB (chronic AVB, closed circles, $n_{\text{dogs}}=6$). **B**, Single cell shortening in myocytes isolated from dogs with chronic AVB (closed circles, $n_{\text{dogs}}=5$, $n_{\text{cells}}=15$), and in cells from control dogs (open circles, $n_{\text{dogs}}=5$, $n_{\text{cells}}=13$).

was significantly larger than in control at frequencies less than 2 Hz ($P < 0.05$), and tended to decrease with increasing frequencies.

Characteristics of $[\text{Ca}^{2+}]_i$ Transients

Action potentials and $[\text{Ca}^{2+}]_i$ transients during whole-cell recording are illustrated in Figure 2. In cells from control dogs, a positive staircase was mostly observed, while in most AVB cells the amplitude of the $[\text{Ca}^{2+}]_i$ transient decreased with increasing frequency. This negative stair resulted from a significant increase in the amplitude of $[\text{Ca}^{2+}]_i$ transients at lower frequencies for cells from chronic AVB, as illustrated by the pooled data in Figure 3A. The increase in peak amplitude at low frequencies was not dependent on a more pronounced increase in APD at the lower frequencies in chronic AVB,²⁰ because it was maintained during stimulation at different frequencies in voltage-clamp mode with a fixed pulse duration (results not shown).

The slower rise following the rapid upstroke of $[\text{Ca}^{2+}]_i$ was more pronounced for AVB dog cells, and the time to peak of $[\text{Ca}^{2+}]_i$ tended to be longer (115 ± 11 ms at 0.25 Hz versus 93 ± 15 ms in control, $P = \text{NS}$). However, significant differences in $[\text{Ca}^{2+}]_i$ were present already during the rapid upstroke (Figure 3B), indicating that early Ca^{2+} release from the SR was larger in AVB cells at the low frequencies. We next examined possible sources of increased Ca^{2+} influx that could provide either a larger trigger for SR Ca^{2+} release and/or the necessary Ca^{2+} influx to increase Ca^{2+} content of the SR.

Properties of the L-type Ca^{2+} Current

I_{CaL} was studied in a separate series of experiments. As illustrated in Figure 4A, the amplitude of the peak currents, measured during 300-ms depolarizing steps, was similar for LV cells from control and from chronic-AVB dogs, and the I-V relations were not shifted. Steady-state inactivation properties, measured for a step to 0 mV, following 2.5-s conditioning pulses to various potentials, were unchanged (Figure 4B).

Ca^{2+} influx via the $\text{Na}^+\text{-Ca}^{2+}$ Exchanger

Ca^{2+} influx via the exchanger in LV cells was measured with I_{CaL} block and with Ca^{2+} release from the SR suppressed with 10 $\mu\text{mol/L}$ ryanodine; pipette $[\text{Na}^+]$ was 20 mmol/L (Figure 5A). During depolarization, $[\text{Ca}^{2+}]_i$ rose slowly; on repolarization $[\text{Ca}^{2+}]_i$ declined, and a $[\text{Ca}^{2+}]_i$ -dependent transient inward current was recorded. This transient inward current was recorded at -45 mV, close to E_{Cl} (-39 mV), and was not sensitive to the Cl^- -channel blocker niflumic acid (100 $\mu\text{mol/L}$), confirming it resulted from Ca^{2+} efflux through the exchanger. The increase in $[\text{Ca}^{2+}]_i$ was significantly larger in cells from chronic AVB than in control cells (Figure 5B). The transient inward current, reflecting the previous increase in $[\text{Ca}^{2+}]_i$ was also significantly larger in chronic AVB (Figure 5C).

Figure 2. – Amplitude and time course of action potentials and $[\text{Ca}^{2+}]_i$ transients during whole-cell recording.

Cells were stimulated with 3-ms depolarizing current pulses, at the indicated frequencies. Representative examples for LV cells from a dog with chronic AVB and a control dog.

Figure 3. – Characteristics of $[\text{Ca}^{2+}]_i$ transients.

A, Frequency-dependence of the peak amplitude of $[\text{Ca}^{2+}]_i$; average data of 11 cells from 5 control dogs (open symbols), and 13 cells from 5 AVB dogs (closed symbols). **B**, $[\text{Ca}^{2+}]_i$ measured at 25 ms following the 3-ms depolarizing current pulse.

Amplitude of $\text{Na}^+\text{-Ca}^{2+}$ -Exchange Currents

We next measured the outward $\text{Na}^+\text{-Ca}^{2+}$ -exchange currents as the Ni^{2+} -sensitive currents, in the same experimental conditions (Figure 6). Consistent with the larger increase in $[\text{Ca}^{2+}]_i$, outward current densities of the exchanger were significantly larger for AVB. In the negative potential range, effects of Ni^{2+} on I_{K1} may pose a problem, and reversal potentials were hard to determine. We studied a number of cells with Cs^+ substituted for K^+ , and found a clear inward exchanger current at the prepulse potential of -50 mV in control cells (-0.51 ± 0.25 pA/pF, $n_{\text{cells}}=5$), whereas we could hardly identify currents in cells from AVB (0.02 ± 0.15 pA/pF, $n_{\text{cells}}=5$), suggesting AVB cells had a more negative reversal potential at resting values of $[\text{Ca}^{2+}]_i$. The current density of the transient inward current on repolarization as a function of the amplitude of the $[\text{Ca}^{2+}]_i$ transient evoked by the depolarizing step was larger for chronic AVB (Figure 6C).

Changes in Ca^{2+} Homeostasis in the Right Ventricle

Similar studies as described above were also performed in cells from the RV; results of the most pertinent protocols are shown in Figure 7. As for cells of the LV, the amplitude of $[\text{Ca}^{2+}]_i$ transients was larger at low frequencies of stimulation (Figure 7A), but the increase was smaller than for the LV cells (compare to Figure 3). I_{CaL} density of RV cells was unchanged with AVB, and comparable to values for the LV (Figure 7B,

Figure 4. – Properties of I_{Cal} .

A, Current densities and IV relations were obtained during 300-ms depolarizing steps from a holding voltage of -70 mV, with a prepulse to -50 mV (top panel); an example of current records from a cell of an AVB dog is shown (middle panel), as well as pooled data (lower panel) for AVB dogs ($n_{\text{cells}}=14$, $n_{\text{dogs}}=4$), and control dogs ($n_{\text{cells}}=12$, $n_{\text{dogs}}=3$). **B**, Steady-state inactivation was studied for a pulse to 0 mV following 2-s steps to various potentials (top panel); an example of currents from a cell of an AVB dog is shown (middle panel), as well as pooled data (lower panel) for AVB dogs ($n_{\text{cells}}=7$, $n_{\text{dogs}}=2$), and control dogs ($n_{\text{cells}}=7$, $n_{\text{dogs}}=2$).

Figure 5. – Ca^{2+} influx via $\text{Na}^+\text{-Ca}^{2+}$ exchange.

A, Examples of current records and $[\text{Ca}^{2+}]_i$ transients during long depolarizing pulses, control cell 110 pF, AVB cell 128 pF. The increasing outward current in the AVB cell at +70 mV is most likely Ca^{2+} -activated Cl^- current since this current was suppressed when the $[\text{Ca}^{2+}]_i$ increase was blocked by Ni^{2+} , and since this was also observed with all K^+ replaced by Cs^+ . **B**, Average increase in $[\text{Ca}^{2+}]_i$ at the end of the pulse of 15 control cells ($n_{\text{dogs}}=7$) and 14 cells from AVB ($n_{\text{dogs}}=6$). **C**, Current density of the transient inward current on repolarization, reflecting the increase in $[\text{Ca}^{2+}]_i$, of the same cells.

Figure 6. – $\text{Na}^+\text{-Ca}^{2+}$ -exchange current densities are larger in chronic AVB.

A, Outward currents were measured by subtraction of currents before and after addition of 2.5 mmol/L NiCl_2 ; records obtained with all K^+ replaced with Cs^+ . Note that Ni^{2+} suppressed the $[\text{Ca}^{2+}]_i$ increase, and had no effect on holding current in the AVB cell (125 pF), but suppressed a small inward current in the control cell (185 pF). **B**, Average density of the Ni^{2+} -sensitive outward current in control ($n_{\text{cells}}=7$, $n_{\text{dogs}}=4$), and in chronic AVB ($n_{\text{cells}}=9$, $n_{\text{dogs}}=5$). In an additional 3 cells for each group, currents were measured at 0, +40 and +70 mV only; values were in the same range as shown for the IV curve. **C**, Density of the transient inward current on repolarization to -45 mV as a function of the $[\text{Ca}^{2+}]_i$ at that time. The full line is the fit to the chronic-AVB data points ($R^2=0.83$), and the dashed line to the data points for control cells ($R^2=0.86$). Statistical analysis separated the 2 groups with $P<0.001$. Data obtained from the experiments shown in Figure 5, for depolarizing steps to +50, +60 and +70 mV.

Figure 7. – RV cell properties in chronic AVB.

A, Average increase in peak $[\text{Ca}^{2+}]_i$ during whole-cell recording in current-clamp mode at different frequencies for control ($n_{\text{cells}}=8$, $n_{\text{dogs}}=3$) and chronic AVB ($n_{\text{cells}}=10$, $n_{\text{dogs}}=4$). **B**, Average current density of I_{Cal} in control ($n_{\text{cells}}=12$, $n_{\text{dogs}}=5$) and chronic AVB ($n_{\text{cells}}=10$, $n_{\text{dogs}}=4$). **C**, Current density of the Ni^{2+} -sensitive outward current in control ($n_{\text{cells}}=7$, $n_{\text{dogs}}=3$) and chronic AVB ($n_{\text{cells}}=8$, $n_{\text{dogs}}=4$). **D**, Density of the transient inward current on repolarization as a function of the $[\text{Ca}^{2+}]_i$ at that time. The linear regression fits to the chronic-AVB data points and to the data points for control cells overlap, ($R^2=0.50$ and 0.64 respectively; the groups could not be separated).

compare to Figure 4). After block of SR release with ryanodine, a significant increase in Ca^{2+} influx via the $\text{Na}^+\text{-Ca}^{2+}$ exchange was also observed for RV cells from chronic-AVB dogs, compared to cells from control dogs (eg, for pulses to +40 mV, $[\text{Ca}^{2+}]_i$ at the end of the pulse was 365 ± 77 nM for AVB ($n_{\text{cells}}=11$, $n_{\text{dogs}}=5$), versus 234 ± 46 nM for control ($n_{\text{cells}}=12$, $n_{\text{dogs}}=6$), but this increase was slightly less than in LV cells ($[\text{Ca}^{2+}]_i$ at +40 mV in LV AVB cells was 423 ± 76 nM). Ni^{2+} -sensitive outward currents were sig-

nificantly larger in RV AVB than in control (Figure 7C), but again slightly less than in LV (at +40 mV, 1.37 ± 0.26 pA/pF in RV AVB versus 1.52 ± 0.24 pA/pF in LV AVB). The transient inward exchanger current at -45 mV as a function of the amplitude of the $[\text{Ca}^{2+}]_i$ transient during the depolarizing step was not significantly increased, and below the values for the LV AVB cells (Figure 7D, contrast with Figure 6C).

Contribution of Reverse-mode $\text{Na}^+\text{-Ca}^{2+}$ Exchange to SR Ca^{2+} Release

We next examined whether $\text{Na}^+\text{-Ca}^{2+}$ exchange could act as a trigger for SR Ca^{2+} release in chronic AVB. To facilitate the study, these experiments were performed with 20 mmol/L NaCl in the pipette; a typical example is illustrated in Figure 8A. The peak amplitude of $[\text{Ca}^{2+}]_i$ tended to be larger in LV AVB cells, and large $[\text{Ca}^{2+}]_i$ transients were observed at positive potentials. However, as we previously observed in guinea-pig cells,²⁴ the voltage dependence of the early increase in $[\text{Ca}^{2+}]_i$ at 25 ms was still bell-shaped, reflecting the voltage dependence of I_{CaL} (not shown). After block of I_{CaL} the upstroke of $[\text{Ca}^{2+}]_i$ was significantly delayed and slower (Figure 8A, traces marked by 'nis'). SR Ca^{2+} release triggered by reverse-mode $\text{Na}^+\text{-Ca}^{2+}$ exchange (ryanodine-sensitive, not shown) was observed more frequently in LV AVB cells, and the amplitude of $[\text{Ca}^{2+}]_i$ transients on average was larger (Figure 8 C,D). In RV cells as well, SR release triggered by $\text{Na}^+\text{-Ca}^{2+}$ exchange was seen more frequently in AVB, but again less than in LV cells (at +10 mV in 2/8 AVB cells, versus 0/8 control cells ($P=\text{NS}$), and at +40 mV in 5/8 AVB cells, versus 2/8 control cells ($P<0.05$), compare to Figure 8C).

We also tested whether the exchanger would contribute to SR Ca^{2+} loading. With 10 mmol/L NaCl in the pipette, and with I_{CaL} blocked, we looked for Ca^{2+} release and oscillations indicative for SR Ca^{2+} overload, during long pulses to positive potentials (Figure 8E). Spontaneous release was observed more frequently in chronic-AVB cells, than in control cells (for steps to +50 mV in 10/12 AVB cells, versus 1/8 control cells, $P<0.01$).

Discussion

Enhanced Contractile Function and SR Ca^{2+} Release in the Dog with Chronic AVB

Measurements of LV dP/dt in vivo indicated that contractile function after 6 weeks of AVB is better than at baseline, and that this improvement is most pronounced at low stimulation frequencies. Whereas interpretation of in-vivo measurements may be complicated by changes in LV diameter,¹⁹ or in autonomic drive, our measurements of cell shortening of the isolated myocytes confirm this finding. The 'inversion' of the $[\text{Ca}^{2+}]_i$ -frequency relation observed in the present study should be contrasted to the findings in human heart failure, where the inversion results from a loss of contraction at the higher rates, with (near) normal function at the low frequencies.^{27,28} The enhanced

Figure 8. – Contribution of $\text{Na}^+\text{-Ca}^{2+}$ exchange to Ca^{2+} release from the sarcoplasmic reticulum.

A, Membrane currents and $[\text{Ca}^{2+}]_i$ transients were recorded during 300-ms depolarizing steps to the indicated potentials (see inset), before and after blocking I_{CaL} with nisoldipine, records marked '+ nis'. The delayed but rapidly rising $[\text{Ca}^{2+}]_i$ transient remaining in the presence of nisoldipine was blocked by ryanodine (not shown). Records obtained in an LV cell from chronic AVB, 88 pF, and from a control dog, 110 pF. **B**, Incidence of Ca^{2+} release triggered by $\text{Na}^+\text{-Ca}^{2+}$ exchange at the indicated potentials. **C**, Average peak $[\text{Ca}^{2+}]_i$ in the presence of nisoldipine for control ($n_{\text{cells}}=9$, $n_{\text{dogs}}=4$) and chronic AVB (closed circles, $n_{\text{cells}}=9$, $n_{\text{dogs}}=4$). **D**, Occurrence of spontaneous Ca^{2+} release during a long depolarizing pulse to +50 mV, in the presence of nisoldipine, LV cell from chronic-AVB dog, 120 pF, and from control dog, 95 pF.

1 Acute AV-block

2 Chronic AV-block

Figure 9 (left page). – Delayed afterdepolarizations and ectopic beats evoked by pacing. At 6 weeks of chronic AVB (right panel), monophasic-action-potential (MAP) duration is increased, and dispersion between LV and RV enhanced compared to the time of creation of AVB (left panel). LV pressure is also higher at chronic AVB. A short pacing train now induces an afterdepolarization manifest in the LV MAP (marked by *), triggering an ectopic beat with a 430-ms coupling interval.

contractile function in chronic AVB maintains cardiac output at the slow idioventricular rate (S.H.M.d.G., PhD thesis). We have recently shown that the functional adaptation occurs in parallel with hypertrophy at the macroscopic and single cell level.²⁰

In animal models of hypertrophy an increase in contraction or $[\text{Ca}^{2+}]_i$ transients was reported for the spontaneously hypertensive rat,²⁹ and no change in contractile function was seen in volume-dependent biventricular hypertrophy of the cat.³⁰ However, more often a depressed function or $[\text{Ca}^{2+}]_i$ was found (eg, References 26 and 31 through 33). Some of these latter animal models may have a relatively early progression to heart failure, complicating the study of the stage of compensated hypertrophy (see also Reference 34). Indeed, a reduction in the amplitude of contraction or $[\text{Ca}^{2+}]_i$ transients at the working heart rates was consistently observed in animal models of heart failure (eg, References 35 through 37). Our current findings are therefore of particular importance as they most likely represent a stage of compensated hypertrophy, before development of overt heart failure, a stage during which in patients, as in this animal model, there is an increased susceptibility to arrhythmias.²

Mechanisms of Increase in Systolic $[\text{Ca}^{2+}]_i$ in Chronic AVB

Analysis of the early phase of the $[\text{Ca}^{2+}]_i$ transients indicates that SR Ca^{2+} release is increased at the lower frequencies. This can be due to an increased Ca^{2+} influx triggering release, or a higher SR Ca^{2+} content. Ca^{2+} influx through the L-type Ca^{2+} channel is unchanged, and can therefore not be responsible for an increase in either trigger Ca^{2+} or SR Ca^{2+} content. In contrast, Ca^{2+} influx through the exchanger is significantly enhanced in chronic AVB. Previous studies in other mammalian species have shown that Ca^{2+} influx through the $\text{Na}^+\text{-Ca}^{2+}$ exchanger is capable of triggering Ca^{2+} release albeit with low efficiency, and contribution of this mechanism in the presence of concomitant influx via L-type Ca^{2+} channels is probably limited (reviewed in Reference 38). Our data in dog myocytes indicate that in this species as well, I_{CaL} is the predominant trigger, necessary to obtain rapidly rising $[\text{Ca}^{2+}]_i$ transients, and the increased Ca^{2+} release in chronic AVB most likely results from an increase in SR Ca^{2+} content, with perhaps a small contribution of an increase in trigger Ca^{2+} . In this respect it is of interest that the increase in the $[\text{Ca}^{2+}]_i$ transient is restricted to the low frequencies of stimulation. In preliminary experiments we found that in AVB dogs the SR Ca^{2+} content, as evaluated during caffeine-induced Ca^{2+} release, indeed tended to be larger at the lower than at the higher frequencies, whereas the reverse was true in control cells. Another hypothesis, which we intend to investigate, is that the local gain of release is increased, as recently proposed.²⁹

Our data support the newly emerging idea that reverse-mode $\text{Na}^+\text{-Ca}^{2+}$ exchange may be important in cardiac remodeling. Increased Ca^{2+} influx via the exchanger, contributing to Ca^{2+} loading of the SR, was recently described in the rabbit heart post-myocardial infarction.³⁹ In human heart failure, the $\text{Na}^+\text{-Ca}^{2+}$ exchanger also appears to be very important in determining the SR Ca^{2+} load,⁴⁰ a notion supported by the finding that increasing Na^+ influx enhances contractile function to a larger extent in myocardium from heart failure patients than from controls.⁴¹

Increased Activity of the $\text{Na}^+\text{-Ca}^{2+}$ Exchanger: Upregulation and/or Changes in Reversal Potential

When directly measured, Ca^{2+} influx via the exchanger was significantly increased in chronic AVB, and outward $\text{Na}^+\text{-Ca}^{2+}$ -exchange currents were more than 3-fold larger in chronic AVB. Such an increase could result from an increase in the number of exchanger molecules, eg, due to higher expression levels, which is then expected to produce a nearly equal increase in inward currents. Although the inward $\text{Na}^+\text{-Ca}^{2+}$ -exchange current for LV cells was larger, the increase was less than for the outward currents, especially in RV cells. One possible explanation is a concomitant negative shift of the reversal potential in chronic AVB, as supported by the presence of a net negative inward current at rest in control, but not in AVB cells. Such a shift could be related to an increase in subsarcolemmal $[\text{Na}^+]$. This hypothesis is attractive, because a small increase in cytoplasmic $[\text{Na}^+]$ has been associated with a preferential increase in Ca^{2+} release at low frequencies of stimulation,^{42,43} and because there is a well-established link between an increase in $[\text{Na}^+]_i$ and the occurrence of spontaneous Ca^{2+} release.^{44,45} We are currently investigating this hypothesis by measuring $[\text{Na}^+]$ in these cells.

Whereas increased exchanger activity has been observed in certain animal models of hypertrophy⁴⁶ and heart failure,¹² this is not always so,^{32,36} suggesting that eventual changes, as in the dog with chronic AVB, represent a specific response, associated with a particular type of Ca^{2+} handling. Consistent with this idea, expression levels of $\text{Na}^+\text{-Ca}^{2+}$ exchange have recently been related to patterns of diastolic dysfunction in human heart failure.⁴⁷

A Link Between Enhanced Ca^{2+} Release, $\text{Na}^+\text{-Ca}^{2+}$ Exchange and Arrhythmogenesis?

In the dog with chronic AVB, as in human ventricular remodeling, increased regional dispersion and prolongation of repolarization, are the substrate for arrhythmias, triggered by ectopic beats and related to afterdepolarizations or local reentry.^{1,2,48} The increase in $\text{Na}^+\text{-Ca}^{2+}$ exchange described in the present study is likely to be an important factor shaping the arrhythmia substrate. Enhanced Ca^{2+} release in steady-state conditions at low frequencies will lead to larger inward $\text{Na}^+\text{-Ca}^{2+}$ -exchange currents during the plateau and the later phase of the action potential; this will be more pronounced in the LV, as $[\text{Ca}^{2+}]_i$ and the inward exchanger current are larger, thus contrib-

uting to regional dispersion. Our current findings may also shed new light on cellular events responsible for the initiation of arrhythmias. Torsades de pointes in the dog with chronic AVB can be evoked in the presence of class III antiarrhythmics, and early afterdepolarizations precede the onset of torsades de pointes.⁴⁹ Although in vitro these early afterdepolarizations are not associated with spontaneous Ca^{2+} release,²⁰ the $\text{Na}^+\text{-Ca}^{2+}$ exchanger may provide the necessary inward current to facilitate depolarizing window Ca^{2+} currents. A more direct role for increased Ca^{2+} release and exchanger current can be inferred for arrhythmias evoked by pacing (in the absence of class III agents), which are dependent on the induction of an enhanced contractile state.⁵⁰ The occurrence of delayed afterdepolarizations, and of ectopic beats, appears to be related to a critical increase in LV dP/dt , which can be obtained in chronic AVB, but not at acute AVB, following a well-defined short pacing train of 8-10 beats, as illustrated in Figure 9. This can be enhanced by ouabain, and suppressed by ryanodine. As the amplitude of the $\text{Na}^+\text{-Ca}^{2+}$ -exchange current will be directly related to the contraction and underlying $[\text{Ca}^{2+}]_i$ transient, this current may provide the dynamic component for the conditional prolongation of the action potential, as well as for the initiation of the ectopic beats during spontaneous Ca^{2+} release. This latter event may be particularly relevant for the arrhythmias occurring during adrenergic stimulation, as previously suggested from in vitro studies.^{51,52} A number of dogs with chronic AVB die suddenly, and preliminary data indicate that this event may be related to changes in autonomic drive. This link is currently being investigated.

Conclusions

An increase in $\text{Na}^+\text{-Ca}^{2+}$ -exchange activity as part of a stimulus-specific ventricular remodeling process has potentially beneficial as well as negative consequences, for contractile as well as electrical function. Enhanced $\text{Na}^+\text{-Ca}^{2+}$ -exchange activity in the dog with chronic AVB may contribute to the adaptation of contractile function by increasing Ca^{2+} release at the lower heart rates. This adaptive mechanism may at the same time contribute to proarrhythmia, by increasing the propensity to Ca^{2+} overload. In addition, increased inward $\text{Na}^+\text{-Ca}^{2+}$ -exchange current at the time of Ca^{2+} release adds to prolongation and dispersion of the action-potential duration, and promotes arrhythmogenic currents during spontaneous Ca^{2+} release.

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Similarities Between Early and Delayed Afterdepolarizations Induced by Isoproterenol in Canine Ventricular Myocytes

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Abstract

Objectives: This study aims at clarifying the role of cellular Ca^{2+} overload and spontaneous sarcoplasmic reticulum (SR) Ca^{2+} release in the generation of early afterdepolarizations (EAD) by isoproterenol. The involvement of a Ca^{2+} -activated membrane current in isoproterenol-induced EAD is investigated.

Methods: Membrane potential and contraction (an indicator of SR Ca^{2+} release) were recorded in canine left ventricular myocytes at pacing cycle lengths (CL) of 300-4000 ms. Threshold concentration for EAD was 20-50 nmol/L isoproterenol. Ni^{2+} (2.0-5.0 mmol/L) was used at normal and high (5.4 mmol/L) $[\text{Ca}^{2+}]_o$ to examine a role of Ca^{2+} current and/or Na^+ - Ca^{2+} exchange ($I_{\text{Na-Ca}}$) in EAD.

Results: In all cells delayed afterdepolarizations (DAD) appeared during isoproterenol. In most ($\approx 70\%$) cells EAD were also generated, which were fast-pacing dependent, occurring only at CL of 400-1000 ms. EAD were always initiated by a delay in repolarization. Early aftercontractions preceded the EAD upstrokes, often occurring without them. They coincided with the initial delays in repolarization. During treatment with isoproterenol, Ni^{2+} and high $[\text{Ca}^{2+}]_o$, EAD and DAD were suppressed despite the continued presence of early and delayed aftercontractions.

Conclusions: Our data indicate that β -adrenergic EAD share a common ionic mechanism with DAD in terms of cellular Ca^{2+} overload and spontaneous SR Ca^{2+} release. β -Adrenergic EAD consist of two phases: (1) a conditional phase coinciding with the onset of an early aftercontraction, often followed by (2) an EAD upstroke. A Ca^{2+} -activated membrane current, likely $I_{\text{Na-Ca}}$, is necessary at least for the initiation of these EAD.

Introduction

Both early (EAD) and delayed afterdepolarizations (DAD) have been incriminated in the generation of cardiac arrhythmias in patients.¹

DAD are generated under conditions that favor accumulation of cytoplasmic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$), cellular Ca^{2+} overload and spontaneous Ca^{2+} release from the sarcoplasmic reticulum (SR). The DAD is caused by a Ca^{2+} -activated inward current during spontaneous SR Ca^{2+} release: the Na^+ - Ca^{2+} exchange current ($I_{\text{Na-Ca}}$)^{2,3} and/or non-selective cation current (I_{NS}).⁴ Together with DAD also delayed aftercontractions are generated and one may assume that spontaneously released Ca^{2+} ions from the SR activate the sarcolemmal inward current and the contractile system simultaneously.⁵

EAD are afterdepolarizations that occur as transient retardations or reversals of the repolarization of the action potential.⁶ Progress and agreement on their ionic mechanism(s) have been meager in comparison with DAD. This relates partly to the variety of conditions during which EAD are generated, indicating that there is not one type of EAD. In general, EAD are generated at slow pacing rates (drive cycle lengths (CL) longer than 1000 ms) and after prolongation of the repolarization. L- and T-type Ca^{2+} current (I_{CaT} and I_{CaL}) as well as Na^+ current (I_{Na}) have been implicated as the currents underlying these EAD¹ (i.e. sarcolemma-dependent mechanisms).

β -Adrenergic receptor stimulation can induce both DAD and EAD. We^{7,8} and other investigators^{9,10} have suggested that cellular Ca^{2+} overload and spontaneous SR Ca^{2+} release are responsible for both afterdepolarizations in this condition via activation of a Ca^{2+} -dependent inward current (i.e. a SR-dependent mechanism).

The present study aims primarily at clarifying the role of cellular Ca^{2+} overload and spontaneous SR Ca^{2+} release in the generation of EAD by isoproterenol in canine left ventricular myocytes. It is hypothesized that if these EAD are generated by a SR-dependent mechanism, early aftercontractions are generated simultaneously. In contrast, if the EAD are generated by a sarcolemma-dependent mechanism, early aftercontractions will be present as a consequence of the EAD and will therefore occur after the EAD upstroke. In addition, block of the currents underlying the EAD will suppress early aftercontractions that are the consequence of the EAD (sarcolemma-dependent mechanism), but will not affect those that are the cause of EAD (SR-dependent mechanism).

Methods

This study was approved by the Animal Studies Subcommittee of the Department of Veterans Affairs, Oklahoma City, USA and the Committee for Experiments on Animals, University of Maastricht, Maastricht, The Netherlands, and conformed with the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health.

Cell-Isolation Procedure

The hearts of adult mongrel dogs anaesthetized with pentobarbital (20-30 mg/kg iv), were quickly excised, washed and a branch of the left-anterior-descending coronary artery was perfused with a cardioplegic solution at 10 °C for 5 minutes. The perfusion was continued at 37 °C with 100 mL of a nominally Ca^{2+} -free standard-buffer solution and with collagenase at low- $[\text{Ca}^{2+}]$ (50 $\mu\text{mol/L}$) for 30 min. After wash-out with enzyme-free low- $[\text{Ca}^{2+}]$ solution the tissue was perfused with the standard-buffer solution containing 1.8 mmol/L $[\text{Ca}^{2+}]$. Finally, the tissue was minced into suspension, filtered and washed five times. Isolated myocytes were stored at 15 °C temperature in either minimum-essential medium or in standard-buffer solution. No electrophysiological differences were observed between cells stored in either solution. The yield of rod-shaped myocytes from one dog heart was always between 60 and 80 %.

Composition of Solutions

The standard-buffer solution contained (in mmol/L): NaCl 145, KCl 4.0, CaCl_2 1.8, MgCl_2 1.0, NaH_2PO_4 1.0, glucose 11, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) 10, pH 7.4 with NaOH at 37 °C and saturated with 100 % O_2 . KCl was set to 8.0 mmol/L in the cold cardioplegic solution. For the disaggregation process, collagenase (0.1 % w/v Type 2, Worthington Biochemical Co., Freehold, NJ or 0.08 % w/v Type A, Boehringer Mannheim, Germany) was used to which was added bovine serum albumin (0.03 % w/v). The storage medium used in part of the cell isolations was minimum essential medium enriched with Hanks' salts (Gibco Laboratories #410-1600EA, Life Technologies, Inc., Grand Island, NY) and with (in mmol/L): NaHCO_3 24, glucose 11, taurine 10, pyruvic acid 2.0, ribose 5.0, allopurinol 0.1, pH 7.4 equilibrated with 5 % CO_2 and 95 % O_2 at 15 °C. Isoproterenol was dissolved in distilled water containing 30 $\mu\text{mol/L}$ ascorbic acid, kept at 5 °C and in the dark. NiCl_2 (2.0-5.0 mmol/L) was added to the standard-buffer solution with or without isoproterenol at a $[\text{Ca}^{2+}]$ of 1.8 or 5.4 mmol/L for inhibition of I_{Ca} and $I_{\text{Na-Ca}}$ (see Results). All of the chemicals used were reagent grade and cell-culture tested (purchased from Sigma Chemical Co., St. Louis, MO). Almokalant was kindly provided by ASTRA, Göteborg, Sweden.

Electrophysiological and Contraction Recordings

A sample of the cell suspension was transferred to a water-jacketed perfusion chamber (0.5 mL) and superfused with standard-buffer solution at a rate of 3 mL/min and at 37 ± 0.5 °C. Cells were allowed to stabilize at CL of 1000 ms for at least 10 minutes before the start of the recordings. Myocytes used for the experiments were selected on the basis of the following criteria: having sharp striations, clear contours, transparent cytoplasm without granulations and blebs, and behaving electrophysiologically normal upon impalement. Cells could be used for experiments until two days after isolation without electrophysiological deterioration. In this period the arrhythmogenic

response of myocytes to isoproterenol was invariably high and it was fully reversible upon washout of the agent.

Membrane-potential recordings and intracellular stimulation were performed with standard glass microelectrodes (3.0 mol/L KCl, 30 to 60 M Ω) connected to a microelectrode amplifier (Axoclamp-2A, Axon Instruments, Inc., Foster City, CA). Myocyte contractions were imaged by a video camera (Panasonic GP-CD60, Panasonic Industrial Co., Secaucus, NJ) connected to an inverted microscope (Nikon, Inc., Melville, NY) and a video edge motion detector (Crescent Electronics, Sandy, UT) allowing for recording at 16-ms temporal resolution.¹¹ The analog output signals were digitized and stored on video tape or computer hard disk for off-line analysis. Membrane potential and cell shortening were recorded simultaneously.

The presence of EAD, appearing as transient retardations and/or reversals of the membrane potential during repolarization, was always confirmed by the reversal of the negative first derivative of the membrane potential¹² towards less negative and/or positive values, respectively. DAD were accepted if their amplitudes were ≥ 5 mV.

Contraction was recorded as an indicator of Ca²⁺-induced Ca²⁺ release and spontaneous Ca²⁺ release from the SR. The amplitude of cell shortening was expressed in μ m. Relative changes of contraction amplitude within one cell (eg, by treatment) are indicated as % of control values. In this manuscript the 'twitch contraction' is that linked to the normal rapid upstroke of the action potential. The 'aftercontraction' follows the twitch contraction either during or after repolarization of the action potential.

The cellular responses to β -adrenergic receptor stimulation were studied in 25 ventricular myocytes from 11 dogs under different conditions.

Statistics

The data are expressed as means $\pm$ SEM. Statistical comparisons were made with the Student's *t* test and the paired *t* test for unpaired and paired data groups, respectively, after testing for the normality of distribution. Differences were considered significant if $P < 0.05$.

Results

The Effect of Isoproterenol on the Ventricular Action Potential and Contraction

In all myocytes a concentration of isoproterenol as low as 20 nmol/L consistently induced increased twitch contractions, followed by the appearance of DAD and delayed aftercontractions. For the generation of EAD, we used the same or slightly higher concentrations (up to 50 nmol/L), inducing EAD in approximately 70 % of all cells tested.

In 10 cells from 6 dogs treated with 20 nmol/L of isoproterenol and stimulated at a CL of 1000 ms, the dome amplitude of the action potential increased from 100 ± 3

to 111 ± 2 mV ($n = 10$; $P < 0.05$). No effect on the resting membrane potential was measured, which was on average -84 ± 3 mV at control. Twitch-contraction amplitudes increased to 683 ± 157 % of the control values. These observations are in line with earlier findings in single myocytes.^{13,14}

Figure 1 illustrates that isoproterenol at low concentration (20 nmol/L) had divergent effects on the action-potential duration at 95 % of repolarization (APD₉₅) of the 10 myocytes: the APD₉₅ either *decreased* (by 47 ± 14 ms, from 274 to 227 ms; $n = 5$; $P < 0.05$) or *increased* (by 13 ± 5 ms, from 242 to 255 ms; $n = 5$; $P < 0.05$). Changes in the corresponding twitch-contraction durations tended to follow the changes in APD₉₅, but were less pronounced. In all 5 myocytes initially responding to isoproterenol with an *increased* APD₉₅, EAD (as well as DAD) were generated upon continued treatment with the agent (Figure 2). We did not see EAD in the absence of DAD. In the 5 myocytes with a *decreased* APD₉₅, only DAD were observed.

After an initial phase with increasing contraction amplitudes, further treatment with isoproterenol rapidly led to an arrhythmogenic response characterized by the induction of EAD, DAD and aftercontractions (as illustrated in Figure 2). Subsequently, DAD-triggered action potentials could occur that could deteriorate into sustained triggered rhythms (data not shown). EAD observed in our experiments did not trigger action potentials nor resulted in sustained triggered rhythms, as was observed earlier.⁹

The enhancement of contraction observed in the initial phase of isoproterenol treatment continued in the presence of afterdepolarizations and aftercontractions (Figure 2; arrow). In all 10 cells larger twitch-contraction amplitudes were intermittently measured in this phase (Figure 2; *) to a maximum of 776 ± 164 % of control amplitudes. This difference between the maximal twitch-contraction amplitudes before vs. after the first appearance of afterdepolarizations and aftercontractions (683 ± 157 % vs. 776 ± 164 % of control, respectively) was significant ($n = 10$; $P < 0.05$). Such large twitch-contraction amplitudes appeared as stimulated contractions (not as aftercontractions; Figure 2).

EAD and Early Aftercontractions Induced by Isoproterenol are Fast-pacing Dependent: Comparison with Almokalant

Isoproterenol-induced EAD were always accompanied by early aftercontractions. Early aftercontractions could occur *without* EAD upstrokes, but always before full repolarization of the action potential. Their gradual appearance after an initial response with increasing contractions and simultaneously with the appearance of DAD suggested a role for cellular Ca^{2+} overload. If so, these EAD and early aftercontractions would be exaggerated by fast pacing and tempered by slow pacing. We tested this in an additional series of 7 myocytes from 4 dogs. Threshold concentrations of isoproterenol used to induce EAD were 20 nmol/L ($n = 2$ cells), 40 nmol/L ($n = 2$) and 50 nmol/L ($n = 3$). We compared this with the rate dependency of EAD induced by the agent almokalant at 3.0 $\mu\text{mol/L}$ in 5 cells from the same 4 dogs. Almokalant is known to possess class-III activity. At nano- to micromolar concentrations it is a specific blocker of the

Figure 1. – The effect of isoproterenol on the action potential and contraction.

Action potentials (lower panel) and contractions (upper panel) are recorded simultaneously at steady-state pacing before (C) and 1 minute after the start of isoproterenol treatment (20 nmol/L; I) and prior to the generation of EAD and/or DAD. There is a differential response of the APD₉₅ to isoproterenol. In myocyte **A**, the APD₉₅ decreases from 251 to 210 ms, while the contraction shortens from 303 to 283 ms. In contrast, in myocyte **B**, the APD₉₅ increases from 251 to 279 ms. The contraction prolongs from 204 to 209 ms. CL = 1000 ms.

rapidly-activating component of delayed-rectifier K⁺ current (I_K),¹⁵ like other pure I_K blockers such as dofetilide and E-4031.¹⁶ These agents delay repolarization and promote EAD induction at slow pacing rates. Pacing CL of 300, 400, 500, 1000, 2000, 3000, and 4000 ms were applied in all cells given either isoproterenol or almokalant. We calculated the number of EAD in 10 consecutive action potentials after ≥ 30 s of steady-state pacing at a given CL.

In Figure 3 it is shown that almokalant-induced EAD occurred only at CL ≥ 2000 ms, with an incidence increasing to 6 EAD per 10 consecutive beats at CL = 4000 ms in these 5 cells. No early aftercontractions were observed with these EAD. The rate dependency of isoproterenol-induced EAD and aftercontractions was completely dif-

Figure 2. – Beat-to-beat induction of EADs and early aftercontractions by isoproterenol. In this myocyte, after the phase with increasing contraction amplitudes, DAD and aftercontractions appear (arrow indicates first action potential with a DAD). Thereafter EAD and early aftercontractions are also generated. Twitch amplitudes are intermittently observed during the phase with DAD and EAD that are larger (*) than the twitch amplitudes prior to the first appearance of DAD and EAD. Typically the increasing twitch amplitudes during systole arise from a stable (ie, non-increasing) diastolic baseline. In this phase there are no triggered action potentials. Isoproterenol: 20 nmol/L; CL = 1000 ms.

ferent. Here, only at CL of 400 to 1000 ms EAD were observed with a peak incidence of 3 EAD per 10 consecutive beats at CL = 500 ms ($n = 7$ cells; Figure 3). Representative action-potential and contraction traces at CL = 500 and 4000 ms are shown in Figure 4. The mutual dependence between APD_{95} and EAD was different for almokalant and isoproterenol. In the case of almokalant, EAD were generated upon an already prolonged action potential. During isoproterenol the APD_{95} was *increased* whenever EAD were (to be) present in consecutive beats, but *decreased* between such beats (Figure 4, upper left panel).

In addition to a difference in rate dependency, there was also a difference in the average take-off membrane potentials of EAD upstrokes in the presence of isoproterenol vs. almokalant. It is shown in the Table that the upstrokes of isoproterenol-induced EAD developed at more negative levels and at broader range. These levels could vary from close to the resting membrane potential to sometimes slightly positive voltages. There was a significant difference between the average take-off potentials of EAD generated at CL = 400 vs. 1000 ms during isoproterenol (Table).

In summary, (1) isoproterenol-induced EAD are fast-pacing dependent; (2) their charge carrier(s) can be activated over a broad range of membrane potentials; and (3) isoproterenol-induced EAD appear to be mechanistically different from almokalant-induced EAD.

Figure 3. – Rate dependency of isoproterenol- vs almokalant-induced EAD in canine left ventricular myocytes.

Pacing CL of 300, 400, 500, 1000, 2000, 3000 and 4000 ms are applied in all cells. Isoproterenol is administered at 20, 40 or 50 nmol/L (until EAD appear; $n = 7$ cells); almokalant at 3.0 mmol/L ($n = 5$ cells). Expressed is the mean number of EAD ($\pm$ SEM) per 10 consecutive beats during steady-state pacing for at least 30 s. Almokalant induces EAD only at CL = 2000, 3000 and 4000 ms; isoproterenol only at CL = 400, 500 and 1000 ms. There is no overlap of the CL at which EAD are induced by these agents.

Early Aftercontractions Precede EAD Upstrokes

To determine the onset of EAD accurately, ie, the time and voltage of the start of the delay in repolarization, we used the first derivative (dV/dt_{rep}) of the membrane potential.¹² This is shown in Figure 5 for beats in which a delay in the repolarization was either followed (Figure 5A) or not followed (Figure 5B) by a frank EAD upstroke.

We examined the temporal relationship between EAD and early aftercontractions in 100 action potentials, as illustrated in the example of Figure 5. The arrows in Figs. 5 A and B indicate the moment of the reversal of dV/dt_{rep} and the same time points on the action potential and contraction. A summary of this temporal comparison is shown in Figure 6. The initial delay in repolarization occurred at 228 ± 22 ms after the upstroke of the action potential at a membrane potential of 1 ± 3 mV. The take off of the upstroke of the EAD came after 271 ± 16 ms at -17 ± 2 mV. The start of the early aftercontraction came at 238 ± 17 ms. This was significantly *earlier* than the start of the upstroke of the EAD (271 ± 16 ms; $P < 0.05$), but not different from the initial delay in repolarization (228 ± 22 ms; $P = \text{NS}$). Early aftercontractions occurred often in the absence of EAD upstrokes (Figure 5 B), but never without the initial delay in repolariza-

Figure 4. – Representative tracings showing generation of EADs at fast pacing rate by isoproterenol and at slow pacing rate by almokalant.

In the presence of isoproterenol (40 nmol/L; left panels) or almokalant (3.0 μ mol/L; right panels) myocytes are stimulated at CL = 500 ms (upper panels) and CL = 4000 ms (lower panels). Note the generation of EAD at CL = 500 ms during isoproterenol and at CL = 4000 ms during almokalant, but not under opposite conditions. Isoproterenol-induced EAD are accompanied by early aftercontractions, almokalant-induced EAD are not. Left Y axes denote membrane potential (mV) in the four panels; right Y axes: cell shortening (μ m); X axes: time (ms). Upper traces denote contraction; lower traces: action potentials.

tion. Generally, their amplitude was larger in the presence than the absence of EAD upstrokes, as illustrated in Figure 5 A and B.

For comparison we evaluated the temporal linking between DAD and delayed aftercontractions measured in 100 action potentials. It was found that the onset of DAD coincided with the onset of delayed aftercontractions in the same way as was found for the start of the delay in repolarization and the onset of early aftercontractions during EAD (Figure 6).

Thus, the time course of isoproterenol-induced EAD is characterized by two phases: (1) a conditional phase¹⁷ (i.e. the initial delay in repolarization); (2) an EAD upstroke. The onset of the early aftercontraction coincides with the conditional phase of the EAD, i.e. significantly earlier than the EAD upstroke.

Generation of early and delayed aftercontractions during inhibition of EAD and DAD by Ni^{2+}

To clarify the relationship between isoproterenol-induced EAD and early aftercontractions we used the cation Ni^{2+} , which in the millimolar range is a non-specific in-

Figure 5. – Temporal relationship between EAD and early aftercontraction.

Contraction (upper trace), first derivative of the membrane potential (middle trace) and action potential (lower trace) is shown for a cycle with an EAD exhibiting a frank upstroke (**A**) and one without an EAD upstroke (**B**). In **A**, a delay in repolarization precedes the upstroke of the EAD, as evidenced by the reversal towards positive levels of dV/dt_{rep} (arrow). This delay is initiated at 240 ms after the upstroke of the action potential and is followed by the EAD upstroke at 310 ms. The start of an early aftercontraction is at 245 ms, at the same moment as the initial delay in repolarization. In **B**, an early aftercontraction appears even without a frank EAD upstroke. The retardation of repolarization, as evidenced by the reversal of dV/dt_{rep} (arrow), is initiated at 313 ms; the start of the early aftercontraction at 309 ms. Note the presence of late-coupled DAD (*) and corresponding aftercontractions in **A** and **B**. The first derivatives of the action-potential upstrokes are artificially truncated.

Table. – The take-off membrane potential of EAD upstrokes during isoproterenol and almokalant in relation to pacing CL

Pacing CL (ms)	Take-off membrane potential (mV)	
	Isoproterenol	Almokalant
400	$-27 \pm 4^*$	
500	-19 ± 3	
1000	-17 ± 2	
2000		-9 ± 5
3000		-14 ± 4
4000		-12 ± 3

Values are averages $\pm$ SEM.
* $P < 0.05$, CL = 400 vs 1000 ms. Isoproterenol, $n = 7$ cells. Almokalant, $n = 5$ cells.

hibitor of the Na^+ - Ca^{2+} exchanger^{3,18} and a blocker of I_{CaL} and I_{CaT} .¹⁹ We reasoned that if the ionic current responsible for the EAD also induces the early aftercontraction, that is if the aftercontraction was secondary to the EAD,²⁰ elimination of the EAD must also eliminate the aftercontraction. On the other hand, if the early aftercontraction is induced by an intracellular mechanism, i.e. spontaneous release of Ca^{2+} from the SR, which also induces the ionic current(s) that produce EAD as we hypothesize, the aftercontraction may occur even when the sarcolemmal currents causing the EAD are blocked and the EAD eliminated. However, aftercontractions due to spontaneous SR Ca^{2+} release would not occur in the presence of a complete block of I_{CaL} because the primary contraction due to Ca^{2+} -induced Ca^{2+} release would also be attenuated. Therefore, we took advantage of the fact that a rise in $[\text{Ca}^{2+}]_o$ can partially overcome the block of I_{CaL} by Ni^{2+} because the blocking effect of Ni^{2+} results from competitive binding to the divalent cation-binding side of the channel.¹⁹ In separate voltage-clamp experiments in 3 myocytes, we confirmed the relief of Ni^{2+} -induced inhibition of I_{CaL} by high $[\text{Ca}^{2+}]_o$ (data not shown).

Ni^{2+} was applied in 5 myocytes from 3 dogs at a concentration of 2.0 or 5.0 mmol/L. As shown for the cell in Figure 7A, following the induction of EAD and DAD by isoproterenol (left panel), Ni^{2+} at normal $[\text{Ca}^{2+}]_o$ (1.8 mmol/L) greatly attenuated the action potential and abolished all twitch contractions, aftercontractions and afterdepolarizations (second from left panel). Thus, Ni^{2+} acted as a Ca^{2+} -channel inhibitor, preventing both Ca^{2+} -induced Ca^{2+} release and spontaneous Ca^{2+} release from the SR not loaded with Ca^{2+} . To offset this effect of Ni^{2+} at least partly, the $[\text{Ca}^{2+}]_o$ was increased to 5.4 mmol/L. Under these conditions there was sufficient I_{CaL} to induce a primary contraction and early and delayed aftercontractions occurred in the absence of EAD and DAD respectively (Figure 7 A, right two panels). The spike-and-dome con-

Figure 6. – Bar graph showing temporal comparison between aftercontractions and afterdepolarizations induced by isoproterenol (20 nmol/L).

A, Onset of aftercontractions vs onset of DAD that are chosen from the recordings in 10 ventricular myocytes. No significant difference in timing is found. **B**, Onset of early aftercontractions vs the initial delays in repolarization (change in dV/dt_{rep}) in action potentials with EAD, and vs the upstrokes of these EAD. The early aftercontractions coincide with the initial delays, but come significantly earlier than the EAD upstrokes. The data are averages $\pm$ SEM for 100 DAD, EAD and their aftercontractions.

121

figuration was still attenuated at this $[Ni^{2+}]_o$. We confirmed these observations in three additional myocytes.

In the other cell showing only DAD, 2.0 mmol/L $[Ni^{2+}]_o$ and high $[Ca^{2+}]_o$ attenuated DAD, but not delayed aftercontractions (Figure 7 B, right panel). The spike-and-dome configuration of the action potential remained at this $[Ni^{2+}]_o$. The washout of Ni^{2+} and high $[Ca^{2+}]_o$ rapidly reversed their actions and afterdepolarizations returned within minutes.

In these Ni^{2+} experiments, early and delayed aftercontractions behaved clearly the same as before. The presence of isoproterenol was essential for their generation: without isoproterenol no aftercontractions.

The step from 1.8 to 5.4 mmol/L $[Ca^{2+}]_o$ during Ni^{2+} and isoproterenol was also essential to generate early and delayed aftercontractions during inhibition of EAD and DAD. Such step in the absence of Ni^{2+} resulted in contracture of the cells within seconds and therefore could not be used as control.

Figure 7. – Generation of aftercontractions without afterdepolarizations in the presence of isoproterenol, Ni^{2+} and high $[\text{Ca}^{2+}]_o$.

Action potentials (lower traces) and contractions (upper traces) are shown during treatment with isoproterenol (20 nmol/L) at CL = 1000 ms. **A**, Generation of DAD, EAD and aftercontractions (left panel). The subsequent addition of 5.0 mmol/L $[\text{Ni}^{2+}]_o$ (second from left panel) abolishes all contractions, aftercontractions and afterdepolarizations. However, an increase of $[\text{Ca}^{2+}]_o$ to 5.4 mmol/L (in the continued presence of isoproterenol and Ni^{2+}) partially restores this effect, as evidenced by the reappearance of twitch contractions and aftercontractions. Note the early aftercontraction without a companion EAD (second from right panel) and the delayed aftercontraction without a DAD (right panel). Similar results were obtained in replicate experiments on 3 cells. **B**, DAD and delayed aftercontraction (onset at 796 ms and 792 ms after the upstroke of the action potential, respectively) are generated (left panel). With the subsequent addition of 2.0 mmol/L $[\text{Ni}^{2+}]_o$ and 5.4 mmol/L $[\text{Ca}^{2+}]_o$ (right panel), the DAD is inhibited, while the aftercontraction remains present starting at 701 ms. In panels **A** and **B**, Ca indicates $[\text{Ca}^{2+}]_o$ and Ni, $[\text{Ni}^{2+}]_o$, both in mmol/L. In all panels DAD, EAD and aftercontractions are marked with *.

In summary, (1) early and delayed aftercontractions can occur independently from EAD and DAD, indicating that spontaneous SR Ca^{2+} release is the primary event; (2) a Ni^{2+} -sensitive inward current, is necessary for both EAD and DAD.

Discussion

Changes in the APD_{95} and Contraction by Isoproterenol

In this study we observed that the APD_{95} of canine left ventricular myocytes either increased or decreased during treatment with 20 nmol/L isoproterenol. Divergent effects of β -adrenergic agonists on the APD_{95} have been reported earlier to occur through direct or indirect modulation of many components that determine the net repolarizing current,^{21,22} however not at the same concentration of the agonist. This duality of isoproterenol responses was often found in myocytes from the same dog heart, which makes it unlikely that our cell-isolation procedure *per se* was responsible for increases of APD_{95} and EAD generation. In addition, these effects disappeared fully upon wash out of isoproterenol.

We did not isolate the cells according to their transmural site of origin. It is known from the work of Antzelevitch et al.²³ that epicardial and endocardial myocytes respond differently to β -adrenergic receptor agonists, i.e. both show action-potential shortening but this is more pronounced in epicardium than endocardium. These investigators have also described a unique population of midmyocardial (M) cells with pharmacological responsiveness different from epicardial and endocardial cells. A weaker I_{K} contributes to a longer action potential in M cells, especially at slow pacing rates.²⁴ Preliminary data from their laboratory show that isoproterenol at 20 nmol/L causes prolongation of the action potential in M cells, but not in epicardial and endocardial myocytes (Dr. C. Antzelevitch, personal communication). This prolongation is greater at more rapid pacing rates, consistent with the possibility that a Ca^{2+} -activated inward current counteracts the weaker outward current to delay repolarization. Isoproterenol was shown to produce even more prolongation and EAD generation in canine left ventricular M cells, but not in epicardial or endocardial cells, when the contribution of I_{K} was further reduced by E-4031.²⁵ Therefore, one possible explanation for the duality of isoproterenol responses in our study could be a heterogeneity of the cell population with M cells having an APD_{95} increase, and epicardial and endocardial myocytes having an APD_{95} decrease.

During β -adrenergic receptor stimulation of ventricular myocytes, increasing twitch contraction amplitudes arose from a stable diastolic baseline, suggesting a beat-to-beat accumulation of Ca^{2+} in the SR without an increment of diastolic $[\text{Ca}^{2+}]_{\text{cyt}}$ (as shown earlier²⁶) and attributed to enhanced uptake of Ca^{2+} by the SR.²⁷ In contrast to the study of Capogrossi et al.,²⁸ the amplitude of twitch contractions of our myocytes was not maximal at the time of the first manifestation of DAD and aftercontractions as in all cells significantly larger amplitudes were intermittently measured during the ar-

rhythmogenic phase of treatment. Our data may indicate that in canine ventricular myocytes the amount of Ca^{2+} loading of the SR necessary for spontaneous Ca^{2+} release is lower than that for maximal inotropy, in line with the computations by Luo and Rudy.²⁹ Species-related differences in intracellular Ca^{2+} handling³⁰ may explain these different findings: in rat myocytes²⁸ the SR is known to work near its capacity for Ca^{2+} sequestration.

Role of Cellular Ca^{2+} Overload in Isoproterenol-Induced EAD

In the present study, EAD and early aftercontractions were generated just after the first appearance of DAD and delayed aftercontractions, often in conjunction with the same action potential. EAD were never observed without DAD. The occurrence of EAD and early aftercontractions was increased by rapid pacing, an experimental condition known to promote cellular Ca^{2+} loading. While the 'optimal' CL for these EAD was around 500 ms, they disappeared at slow pacing (CL $\geq$ 2000 ms) and as such behaved completely different from the EAD induced by almolant. These links between the appearance of EAD and DAD, and between EAD and fast pacing strongly support our hypothesis that isoproterenol-induced EAD are related to cellular Ca^{2+} overload.

Earlier, we have demonstrated the simultaneous generation of EAD and DAD in ventricular myocytes under different conditions of Ca^{2+} loading.³¹ In a recent preliminary study,³² the development of EAD in ventricular cells and Purkinje fibers exposed to E-4031, was shown to be promoted by the acceleration of stimulation from initially slow rates, indicating that increased $[\text{Ca}^{2+}]_{\text{cyt}}$ could also be involved in the generation of those EAD.

Role of Spontaneous SR Ca^{2+} Release in Isoproterenol-Induced EAD

In this study, we recorded contraction as a physiological marker of Ca^{2+} -induced and spontaneous Ca^{2+} release from the SR at relatively high temporal resolution (16 ms). The onset of early aftercontractions coincided with the initial delays in repolarization during EAD, on average more than 30 ms before the EAD upstrokes. It is recognized that contraction can lag behind $[\text{Ca}^{2+}]_{\text{cyt}}$ in a complex manner.³³ As but one example, Berlin et al.³⁴ showed that during DAD in rat ventricular myocytes, the Ca^{2+} aftertransient coincided with I_{T1} and both preceded the aftercontraction by 60 to 90 ms.³⁴ If we apply such delay to our recordings, it implies that the underlying Ca^{2+} aftertransients occurred 60 to 90 ms earlier than the early aftercontractions and even more early than the EAD upstrokes.

It has been proposed that early aftercontractions are the result of Ca^{2+} -induced Ca^{2+} release and that sarcolemmal 'window' I_{CaL} (underlying the EAD) triggers this Ca^{2+} -induced Ca^{2+} release.^{20,35} However, our observation of the temporal coincidence between the onset of early aftercontractions and the conditional phase of EAD (but not the EAD upstroke) suggests the opposite, namely that spontaneous SR Ca^{2+} release activates I_{T1} counteracting the repolarizing currents. This hypothesis predicts that aftercontractions based on spontaneous SR Ca^{2+} release can occur without EAD.

Our experiments with Ni^{2+} show that EAD can be eliminated completely without suppression of the early aftercontractions, demonstrating conclusively that the aftercontractions cannot all be secondary to the currents that induce EAD. Under these conditions, the aftercontractions cannot be due to Ca^{2+} -induced Ca^{2+} release from a surge of I_{CaL} that simultaneously also generates the EAD, as hypothesized by others.^{20,29,35} Since the aftercontractions must be due to Ca^{2+} release from the SR that is not induced by transsarcolemmal Ca^{2+} current, the Ca^{2+} release responsible for the aftercontractions must be by definition spontaneous. Spontaneous Ca^{2+} release has been accepted as the basis for DAD and delayed aftercontractions. However, the precise mechanisms for spontaneous Ca^{2+} release are unclear. Although our observations do not exclude the possibility that some EAD with take-off membrane potentials more positive than -35 mV are generated by reactivation of I_{CaL} , they establish that some aftercontractions and their associated EAD are independent of I_{CaL} . With respect to the specific identity of the current carrier for the EAD associated with spontaneous Ca^{2+} release, our experiments are not as conclusive because unknown effects of Ni^{2+} may exist on other currents which may be candidates for generating EAD. The Ni^{2+} experiments demonstrate that aftercontractions are primary and not secondary and thus support the inference of a Ca^{2+} -activated inward current in EAD.

With the use of fluorescent Ca^{2+} indicators, Ca^{2+} aftertransients of relatively large amplitude have been observed during isoproterenol-induced EAD.^{10,35} Yamada and Corr described these transients as multiple peaks of $[\text{Ca}^{2+}]_{\text{cyt}}$ following the action-potential upstroke.¹⁰ The temporal relationship between EAD and Ca^{2+} aftertransients could not be determined in these experiments, due to the low time resolution (40 ms) of the techniques used.³⁵ Ca^{2+} aftertransients were inhibited by ryanodine, thus identifying the SR as the source of Ca^{2+} .¹⁰ However, whether these transients were the result of Ca^{2+} -induced Ca^{2+} release or spontaneous Ca^{2+} release from the SR could not be distinguished with the use of ryanodine.

The trigger for 'spontaneous' Ca^{2+} release during EAD and DAD is unknown. β -Adrenergic receptor stimulation has been shown to augment the removal of Ca^{2+} from the cytoplasm both into the extracellular space and into the SR. This suggests that the triggering of 'spontaneous' Ca^{2+} release may be linked to a high level of Ca^{2+} in the SR. Recently, Ca^{2+} sparks have been observed, which have been presented as the elementary phenomenon of Ca^{2+} -induced Ca^{2+} release.³⁶ It has been demonstrated that the fractional release of Ca^{2+} from the SR is increased with an increase of the SR Ca^{2+} content.³⁷ Therefore, it could be possible that in the presence of isoproterenol Ca^{2+} sparks are augmented both in amplitude and in number and that they trigger a general SR Ca^{2+} release as the result of their spatial and temporal summation, which has been shown to occur in myocytes loaded with Ca^{2+} by high $[\text{Ca}^{2+}]_0$.³⁸ In addition, the spontaneous Ca^{2+} release inducing early aftercontractions could be triggered by Ca^{2+} being removed from the myofilament space after the twitch contraction and encountering a suprathreshold gradient of Ca^{2+} over the SR membrane.

Isoproterenol-Induced EAD Are Initiated by I_{Na-Ca}

Based on the results of our study we conclude that cellular Ca^{2+} overload and spontaneous SR Ca^{2+} release are responsible for isoproterenol-induced EAD via activation of a Ca^{2+} -dependent inward current. This current appeared to be Ni^{2+} sensitive. Ni^{2+} is a blocker of I_{CaL} , I_{CaT} and I_{Na-Ca} . It has already been discussed that I_{CaL} or I_{CaT} are unlikely to be involved in the *initiation* of EAD and DAD, leaving I_{Na-Ca} as a serious candidate.

A role for I_{Na-Ca} is also supported by our observation that EAD could be activated over a broad range of membrane potentials. Schouten et al.³⁹ demonstrated that I_{Na-Ca} is present throughout repolarization. They indicated that an increase in the Ca^{2+} transient would enhance inward I_{Na-Ca} during the action potential and delay repolarization, a point confirmed in the comprehensive mathematical model of the electrical activity of the ventricular myocyte by Luo and Rudy.²⁹ These mechanisms could explain why a modest action-potential prolongation was the initial response in myocytes ultimately generating EAD during isoproterenol.

At least one other candidate for the Ca^{2+} -activated inward current has to be considered, namely I_{NS} .⁴ However, the presence of Ca^{2+} -activated non-selective cation channels has not been demonstrated in canine ventricular myocytes, while the contribution of I_{NS} to I_{T1} in other species is not consistent (eg, Reference 40). Moreover, on the basis of experiments with the cation Sr^{2+} , Niggli⁴¹ found that Ni^{2+} inhibited I_{T1} generated by the Na^+-Ca^{2+} exchanger, whereas it did not block current through the Ca^{2+} -activated non-selective cation channel. Our observation that Ni^{2+} completely suppressed both DAD and EAD suggests that I_{NS} was not involved. It remains possible however that Ni^{2+} blocks some yet-unidentified Ca^{2+} -activated inward current that could contribute to EAD.

Thus, our present results indicate that I_{Na-Ca} plays an important role at least in the initiation of isoproterenol-induced EAD. The initial delay in repolarization characterizing the conditional phase of these EAD may set the stage for other inward currents to become activated. In this way, I_{CaL} could also be activated, not as the original cause of the EAD, but rather as the consequence of the depolarization initiated by I_{Na-Ca} .

Possible Clinical Implications

Our results in isolated myocytes suggest that β -adrenergic receptor stimulation of the working myocardium could be arrhythmogenic by two distinct mechanisms. With intracellular Ca^{2+} overload, DAD appear that can induce triggered action potentials and sustained triggered rhythms. The EAD do not trigger new action potentials simply because at the membrane potential of their occurrence peak I_{Na} cannot be activated. However, the EAD could induce electrical heterogeneity by causing a variable prolongation of repolarization. Regional differences of repolarization (ie, dispersion) favor reentry^{42,43}.

The present study supports a role for β -adrenergic receptor stimulation in facilitating the onset of ventricular tachyarrhythmias on the basis of EAD generation and electrical heterogeneity in the idiopathic long QT syndrome.^{44,45} In addition, the con-

traction abnormalities that accompany EAD generation could provide the cellular basis for the wall motion abnormalities of patients suffering from this syndrome.⁴⁶ The alleviation of both the electrical and mechanical abnormalities of patients with the long QT syndrome by Ca^{2+} channel blocking agents suggests that abnormal cellular Ca^{2+} handling is the common denominator. Ca^{2+} -channel blockade would prevent or diminish the accumulation of intracellular Ca^{2+} and thus inhibit the generation of EAD and aftercontractions by preventing spontaneous Ca^{2+} release from the SR.

Conclusions

It is concluded that afterdepolarizations and aftercontractions induced by isoproterenol can occur during systole and diastole. Their simultaneous occurrence indicates a common underlying mechanism, likely spontaneous SR Ca^{2+} release. The observation that the onset of early aftercontractions precedes the upstroke of EAD also supports the role of spontaneous SR Ca^{2+} release and indicates the involvement of a Ca^{2+} -activated current, most likely $I_{\text{Na-Ca}}$. The inhibitory effect of Ni^{2+} on both EAD and DAD, but not on their simultaneous aftercontractions, provides further support for the involvement of spontaneous Ca^{2+} release and $I_{\text{Na-Ca}}$ in afterdepolarizations induced by β -adrenergic receptor stimulation.

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General Discussion

Part I of the **General Discussion** (Chapter 7) will deal with the specific electrical alterations in the dog with chronic complete atrioventricular block and how these could lead to arrhythmias in the animal model.

In **Part II** of the **General Discussion**, (Chapter 8) the new information from this thesis is put into a broader perspective, from cell to clinic, and a new view is formulated on Ca^{2+} -dependent mechanisms of early afterdepolarizations and their significance for the initiation and treatment of torsades de pointes.

Altered Electrical Properties in the Dog With Chronic Complete Atrioventricular Block: The Substrate and the Trigger for Torsades de Pointes

Content

1. Introduction
2. The Substrate
3. The Trigger
4. Possible Stimuli for Electrical Remodeling
5. Where to Go from Here?
6. References

1. Introduction

For long, it has been recognized that the mammalian ventricular myocardium is characterized by regional differences in electrical properties. Experimental studies in the 1950s and 1960s demonstrated nonuniformity in the recovery from excitation in the endo- *versus* epicardium,^{1,2} and on the epicardial surface (anterior wall).³ van Dam and Durrer⁴ considered that the differences in refractoriness between the endo- and epicardial layers would not necessarily reflect the temporal sequence of repolarization across the transmural ventricular wall. They employed needle electrodes to determine the time course of excitability within the ventricular wall of the intact dog heart ('*mid-antrolateral portion of the left ventricle*') and showed that the recovery from excitation was not uniform for all layers, but was *shorter* in the '*middle layers than in the subendocardial and outer wall*'.⁴ Mendez et al⁵ were among the first to report on electrical differences between Purkinje fibers and muscle tissue, on the basis of which reentrant activity in the Purkinje fiber-muscle junction could later be explained.⁶ Since that time, the complex geometry and high degree of heterogeneity of the action-potential duration (APD) of the Purkinje fiber-muscle junctional region have often been implicated in cardiac arrhythmogenesis under various conditions.⁷⁻¹¹ With the advent of the micro-electrode technique and whole-cell voltage clamp, it became possible to study the electrical properties of the myocardium at the level of the action potential and its underlying membrane currents. These studies indicated a cellular, ionic basis for the previously-found differences in refractoriness between the epicardium and endocardium.¹²⁻¹⁶ In 1991, Sicouri and Antzelevitch¹⁷ described a unique population of cells (termed M-cells) in the deep subepicardium of the canine ventricles with electrophysiological features remarkably different from those of epi- and endocardium. Microelectrode recordings in isolated tissue preparations revealed an increased maximal rate of rise of the action-potential upstroke, a longer APD, and a more pronounced action-potential prolongation upon slowing of the pacing rate of the M-cells.¹⁷ Likewise, the responses of M-cells to class IA¹⁸ and class III agents were more pronounced. M-cells appeared to be widely distributed (base to apex) in the intramural left (LV) and right ventricle (RV), and also in the interventricular septum.^{17,19,20} Subsequent isolated-tissue²¹ and single-cell work^{22,23} confirmed the presence of M-cells in the human ventricular wall. To explain the longer APD of M-cells compared to endo- and epicardial cells, the contribution of a weaker slowly activating delayed rectifier K⁺ current (I_{Ks})^{24,25} and a larger late Na⁺ current (I_{Na})²⁶ were inferred. The three layers exhibited no differences in the rapidly activating delayed rectifier K⁺ current (I_{Kr}) and the inward rectifier K⁺ current (I_{K1}).^{25, 27} Recent results show that the transmural differences in APD are maintained in arterially-perfused wedge preparations (with a higher integrity and coupling of the myocardial cells), but they are quantitatively smaller due to electrotonic interaction.²⁸ Similar experiments provided evidence that the voltage gradients between the M region and the epi- and endocardium contribute prominently to the inscription of the electrocardiographic T wave under normal conditions and to the

arrhythmogenic transmural dispersion of repolarization involved in torsades-like polymorphic tachyarrhythmias during perfusion with class III agents.²⁹ The results of this²⁹ and other studies³⁰⁻³³ implicate an important role for M-cells in ventricular tachyarrhythmias, notably torsades de pointes. Because of these explicit features, we chose to isolate cells from the midmyocardium in most of the studies of this thesis.

A controversy currently discussed in the literature is if and to what extent M-cell activity can be recorded in the intact, beating heart under normal and pathophysiological circumstances, and whether there is an inhomogeneous transmural gradient for ventricular repolarization.^{34,35}

It is of great importance to identify the regional, electrical characteristics of the ventricular myocardium under normal conditions, as these may show variable responses during patho(physio)logical conditions. In ventricular hypertrophy and failure of different origin, action-potential prolongation is a very consistent electrophysiological alteration,³⁶⁻³⁸ which can often occur at different degrees in different regions.³⁹⁻⁴² The resultant increased dispersion of repolarization can predispose to ventricular arrhythmias and sudden cardiac death.⁴³

Biventricular hypertrophy and electrical remodeling are prominent features of myocardial adaptation in the dog with chronic complete atrioventricular block (AVB), and they are associated with an enhanced susceptibility to acquired torsades de pointes.⁴⁴ The cellular results of this thesis indicate that the electrophysiological substrate for proarrhythmia is characterized by: (1) intrinsic disturbances of repolarization with prolonged action potentials at baseline; (2) pronounced regional dispersion of repolarization; and (3) increased susceptibility to action-potential prolongation and generation of early afterdepolarizations during administration of class III antiarrhythmic agents. In addition, the data indicate that alterations in cellular Ca^{2+} homeostasis cause an increased propensity to Ca^{2+} overload and spontaneous Ca^{2+} release from the sarcoplasmic reticulum. These events could trigger ventricular ectopic beats via early and delayed afterdepolarizations.

2. The Substrate

We have demonstrated that, in the normal dog heart, differences in action-potential configuration and APD are present between the LV and RV free wall. This was established *in vivo* with monophasic-action-potential catheters applied to the endocardium and *in vitro* with microelectrodes in midmyocardial cells (Chapter 2). The ionic basis of these action-potential characteristics is related to a different functional expression of the K^+ currents I_{TO} and I_{Ks} between the ventricles (Chapter 2), and it is likely that also other membrane currents are involved. In the state of chronic AVB, the pronounced interventricular dispersion of repolarization appears to be based, at least partly, on an exaggeration of the action-potential differences at control: the spike-and-dome configuration is maintained and the difference in APD between LV and RV

myocytes has significantly increased (Chapter 3). Administration of the class III agent almokalant increases the APD to a similarly high level in both ventricles, thus unmasking the presence of intrinsic repolarization disturbances in both myocyte populations. In contrast to the APD pattern, the cellular growth response is larger in the RV (+23% versus control) than in the LV (+13%), which may reflect a greater impact of the altered hemodynamics on that chamber after the transition from SR to idioventricular rhythm and also indicates that there is no simple relation between cell size and APD.

To explain the altered electrical properties in the chronic, hypertrophic state of AVB, we hypothesized three scenarios: (1) the ionic currents contributing to electrical remodeling were similar in both ventricles, but because the substrates were different at control (electrical nonuniformity between RV and LV), the impact of these contributions would be different; (2) the ionic currents to remodeling were similar in both ventricles, but their functional alterations occurred at different degrees; (3) the ionic currents contributing to remodeling were different in the two ventricles.

To learn more about the actual scenario, we performed whole-cell voltage clamp and $[Ca^{2+}]_i$ measurements. In the RV there was a prominent decrease of both I_{K_S} (-55%) and I_{K_r} (-45%; Chapter 4), and yet the action-potential prolongation was minimal at baseline. Only after administering almokalant to the RV myocytes, a pronounced, pathological prolongation was observed and this suggested that the residual fraction of I_{K_r} played a major role in their repolarization. In the LV there was a prominent prolongation of only I_{K_S} (-50%) and here the action-potential prolongation was already prominent at baseline. Next to I_{K_S} and I_{K_r} , the magnitude of I_{K_1} was unaltered and so was the interventricular difference of I_{T_O} . These paradoxical findings led to the following conclusions: (1) at control, the *repolarization reserve* of RV midmyocardial cells (ie, their intrinsic, ionic capacity to maintain a fast repolarization) was larger than of LV companions; and (2) the altered action-potential properties in chronic AVB, were based on the downregulation of K^+ currents, but most likely also on the alteration(s) of other membrane currents involved in the repolarization.

Important new insights were also provided by the studies on $[Ca^{2+}]_i$ transients and Ca^{2+} -dependent ionic currents (Chapter 5). The $[Ca^{2+}]_i$ transients of chronic-AVB myocytes appeared significantly larger than those of control cells at low stimulation rates relevant for the in-vivo idioventricular rhythm, and this was more pronounced in the LV than RV. Inward I_{Na-Ca} was also larger and was due to the larger $[Ca^{2+}]_i$ transients as well as larger current densities per given $[Ca^{2+}]_i$. The latter was again more pronounced in the LV than RV. The activation and inactivation characteristics of I_{CaL} were not modified. Thus, altered Ca^{2+} homeostasis in chronic AVB accentuated the interventricular dispersion of repolarization by the differential upregulation of inward I_{Na-Ca} , causing more pronounced action-potential prolongation in the LV than in the RV.

If we consider the ensemble of ionic currents responsible for the repolarization of ventricular cells at control (see Figure 3 in Chapter 1) and their alterations in chronic AVB, none of the three scenarios hypothesized holds true exclusively. Rather, the three

occur in concert. Furthermore, it is very well possible that other ionic currents than presently investigated contribute to the pronounced regional dispersion of repolarization. Here we can think of the late I_{Na} , the I_{Na-K} and additional ionic currents known to be present under normal or pathophysiological circumstances.

What is the significance of increased interventricular dispersion of repolarization for the genesis of torsades de pointes? We believe that the observed differences in action-potential configuration and APD between the ventricles reflect the existence of dispersion in more closely juxtaposed regions, such as the transmural ventricular wall and/or the interventricular septum. Preliminary in-vivo data from our laboratory indicate that the transmural interventricular septum is a region of particular interest in this regard, because relatively large repolarization gradients are present in chronic AVB.^{45,46} Arrhythmogenic voltage gradients can arise in such areas of myocardium when the differences in refractoriness are sufficiently large to cause reexcitation.⁴⁷⁻⁴⁹ The resultant extrasystolic activity can initiate torsades de pointes.³⁰ Under these circumstances the generation of early afterdepolarizations may facilitate proarrhythmia by an acute exaggeration of the spatial and temporal electrical heterogeneity favoring reentry.

3. The Trigger

Ventricular ectopic beats are important for the initiation of torsades de pointes. We have just discussed that phase-2 reentry can be a mechanism of extrasystolic activity in the myocardium of dogs with chronic AVB on the basis of increased regional dispersion of repolarization and/or early afterdepolarizations. For abnormal impulse generation of this kind to occur, a critical number of integrated myocytes is necessary. The present studies bring forward that other forms of abnormal impulse generation, which arise in the single cell, must also be considered. Chronic-AVB myocytes exhibit an increased propensity to Ca^{2+} overload and spontaneous Ca^{2+} release from the sarcoplasmic reticulum. Together with the enhanced Na^+-Ca^{2+} -exchange activity these events facilitate the generation of afterdepolarizations and triggered action potentials. In-vivo experiments in chronic-AVB dogs confirm this notion: significant increases in the LV-pressure rise during short pacing trains are coupled to a steepening of the diastolic slope on the monophasic-action-potential signal and to the induction of ectopic beats, both of which are less pronounced or absent in acute AVB (Chapter 5).⁵⁰ The in-vivo relevance of afterdepolarizations is further suggested by the observation that increased adrenergic stimulation is frequently involved in the initiation of torsades de pointes.⁵¹⁻⁵⁵ As discussed in Chapters 6 and 8, intense β -adrenergic receptor stimulation causes spontaneous Ca^{2+} release from the sarcoplasmic reticulum that can occur during systole and diastole, thus giving rise to both early and delayed afterdepolarizations. The simultaneous occurrence of both membrane responses in relation to the same action potential often precedes a run of torsades de pointes.

Finally, within the context of abnormal impulse generation, abnormal automaticity should not be overlooked as a possible arrhythmogenic mechanism in the dog with chronic AVB, although this was not investigated. In aged hypertensive rats, the expression of the hyperpolarization-activated pacemaker current I_f is linearly related to the severity of myocardial hypertrophy, and its upregulation was implicated as a cellular mechanism for ventricular arrhythmias in this species.⁵⁶ In trabeculae from failing human hearts, automaticity was observed under experimental conditions that mimicked the altered extracellular environment in vivo.⁵⁷

4. Possible Stimuli for Electrical Remodeling

In the diagram, possible stimuli for the altered electrical properties of the dog with chronic AVB are highlighted. If we consider the cascade of events induced by the creation of AVB, then critical factors are bradycardia, increased myocardial stretch and altered ventricular activation. In addition, neurohumoral activation and possibly myocardial ischemia will involve. Bradycardia is associated with an increased APD and prolonged diastolic intervals. Both affect the kinetics of I_{Ks} and I_{Kr} (and all other currents), and may cause definite current changes at chronic duration. Myocardial stretch is the trigger of numerous intracellular signaling pathways that eventually lead to cellular hypertrophy.⁵⁸ Of the second messengers activated, protein kinases A and C have a direct effect on I_{Ks} ⁵⁹ and may alter channel function during long-term modulation. In addition, cell growth could be disproportionate to channel upregulation leading to decreases in current density. Altered ventricular activation is known to cause altered transmural gradients of repolarization and typical T-wave changes in experiments with dogs designed to study long-term cardiac memory.⁶⁰ Rosen et al^{60,61} discussed that I_{Ks} and I_{Kr} could play an important role under these circumstances. It is conceivable that the idioventricular rhythm in chronic AVB mimics the situation in long-term memory to some degree. Finally, we cannot rule out that a relative ischemia occurs during bradycardia and the development of hypertrophy. Subendocardial hypoperfusion during exercise has been described in dogs with volume-overload hypertrophy due to aortocaval fistulas.⁶² It was shown that ischemia reduces I_{Ks} ,⁶³ and also I_{Kr} .⁶⁴

The altered Ca^{2+} dynamics can be explained, at least partly, by the upregulation of Na^+ - Ca^{2+} -exchange activity. Outward Na^+ - Ca^{2+} -exchange currents were severalfold larger in chronic AVB and this was the cause of an increased $[Ca^{2+}]_i$ loading and release from the sarcoplasmic reticulum. The increased exchanger function could result from an increase in the number of exchanger molecules, eg, due to higher expression levels. Another explanation could be a negative shift of the reversal potential. Such a shift could be related to an increase in subsarcolemmal $[Na^+]$, possibly due to an altered function of the Na^+ - K^+ pump. These hypotheses are currently being investigated in close collaboration with Prof. Dr. F. Verdonck, University of Leuven at Kortrijk, Belgium.

Induction of complete atrioventricular block (AVB):

Hypotheses on the stimuli for electrical remodeling

Figure. – Schematic diagram of the possible stimuli for electrical remodeling in the dog with chronic complete atrioventricular block.

5. Where to Go from Here?

The dog with chronic AVB is an excellent model for the study of ventricular arrhythmias in cardiac hypertrophy. Now that we have established that the process of electrical remodeling is determined by intrinsic changes in membrane currents, it is particularly relevant to know their time dependency and reversibility. The longitudinal design of such studies is a prerequisite to obtain the most consistent information on time-dependent alterations that generate proarrhythmia and to identify the responsible pathogenetic factors. Molecular studies (in-situ hybridization, histochemical studies, analyses of mRNA and protein levels) on myocardial biopsies can contribute importantly to this identification. It is not that we have an exclusive interest for the dog with chronic AVB. Rather, the aim is to compare insights from this model with those from other models of heart disease and to implement all into a framework of common pathophysiologic pathways. Ultimately, this may facilitate the understanding and treatment of proarrhythmic responses in the hypertrophied human heart.

The combined findings of an enhanced susceptibility to acquired torsades de pointes, the (supposed) adrenergic dependence of spontaneous episodes of torsades de pointes, the typical T-wave patterns during prolonged QT intervals, and the reduction of I_{Ks} in this animal model closely resemble the clinical characteristics of the LQT1 form of the human congenital long-QT syndrome. In the dog with chronic AVB, this provides a unique opportunity to investigate the adrenergic dependence of torsades de pointes and sudden cardiac death under controlled conditions.

6. References

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Progress in the Understanding of Cardiac Afterdepolarizations and Torsades de Pointes: Time to Revise Current Concepts

This chapter is at the basis of a review article in preparation by:

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Content

1. Introduction
2. Cellular mechanisms of Ca^{2+} homeostasis and their relation to delayed and early afterdepolarizations
 - 2.1 Ca^{2+} homeostasis in normal cardiac cells
 - 2.2 Differences of $[\text{Ca}^{2+}]$ between the bulk cytoplasm and the subsarcolemmal space
 - 2.3 Cellular Ca^{2+} (over)load and spontaneous Ca^{2+} release from the SR
 - 2.4 Ionic mechanisms of DADs
 - 2.5 Ionic mechanisms of EADs
 - 2.6 Evidence for $[\text{Ca}^{2+}]_{\text{cyt}}$ -dependent mechanisms of EADs
3. Afterdepolarizations and arrhythmogenesis in myocardial tissue and the intact animal heart
 - 3.1 EADs in multicellular preparations: consequences for arrhythmogenesis
 - 3.2 Simultaneous appearance of DADs and EADs in the intact heart
 - 3.3 EADs occurring in conditions associated with myocardial Ca^{2+} overload
4. Clinical management of arrhythmias in the congenital and acquired long-QT syndromes
5. Conclusions
6. References

1. Introduction

Afterdepolarizations are oscillations of the membrane potential that depend on the preceding action potential for their generation.¹ Within single cardiac cells, afterdepolarizations can give rise to new action potentials when they reach a critical threshold membrane potential for activation of a major depolarizing cation current. This form of abnormal impulse generation is called '*triggered activity*'.² Afterdepolarizations can also augment electrical heterogeneity in regions of neighboring cells, which can lead to the formation of new action potentials via electrotonic interaction between regions that have recovered from refractoriness and those that are still inexcitable.³ Although, by definition, the latter mechanism is not a form of abnormal impulse generation, the occurrence of afterdepolarizations appears to be of pivotal importance for arrhythmogenesis under many of these circumstances. Thus, afterdepolarizations are of importance for the generation of arrhythmias. Experimental or clinical interventions aimed at preventing or attenuating their occurrence are therefore powerful antiarrhythmic tools.

Two types of afterdepolarizations can be distinguished: delayed (DADs) and early afterdepolarizations (EADs). DADs have been defined as '*oscillations in membrane potential that occur after repolarization of an action potential and that are caused by that action potential*'.⁴ EADs appear during the action potential and have been defined as '*oscillations at the plateau level of membrane potential or later during phase 3 of repolarization*'.⁴

The occurrence of DADs in the myocardium has been related to changes in contraction and Ca^{2+} handling,⁵⁻¹⁷ and it is generally accepted that DADs are caused by transient inward currents (I_{TI}) evoked by spontaneous Ca^{2+} releases from the sarcoplasmic reticulum (SR) under conditions that favor accumulation of cytoplasmic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) and cellular Ca^{2+} overload.^{4,18}

Progress and agreement on the mechanisms of EADs have been meager in comparison with DADs. With respect to the upstroke phase of EADs there is general consensus that L- and T-type Ca^{2+} current (I_{CaL} and I_{CaT}), and Na^{+} current (I_{Na}) can be major charge carriers (ie, *sarcolemma-dependent mechanisms for EADs*).^{19,20} Less agreement has been reached on the ionic basis of the so-called '*conditional phase*'. This is the transient perturbation of the normal course of repolarization that precedes the upstroke of most types of EADs. Slowed inactivation and/or reactivation of I_{CaL} and I_{Na} are contributors to this phase, but it is now becoming clear that the $[\text{Ca}^{2+}]_{\text{cyt}}$ -activated Na^{+} - Ca^{2+} -exchange current ($I_{\text{Na-Ca}}$) also plays a significant role in many cases (ie, *$[\text{Ca}^{2+}]_{\text{cyt}}$ -dependent mechanisms for EADs*). Based on our own work and accumulating evidence in the literature, such a role is not only restricted to EADs related to Ca^{2+} overload and spontaneous Ca^{2+} release from the SR, but applies also to EADs that occur in the setting of less extreme Ca^{2+} loading.

Whereas numerous studies have accentuated the differences between DADs and EADs, we and other groups have often described similarities between the two. In Table 1, a number of experimental conditions associated with the generation of both

DADs and EADs in transmembrane and/or monophasic-action-potential (MAP) recordings are categorized. A first argument favoring the contribution of $[Ca^{2+}]_{cyt}$ -dependent mechanisms for EADs is based on the observation that, dependent on the mode of induction, DADs and EADs can appear jointly in consecutive action potentials or even in relation to the same action potential. A second argument for $[Ca^{2+}]_{cyt}$ -dependent mechanisms derives from the dynamic cellular responses to rate acceleration and adrenergic-receptor stimulation, known clinically to be involved in the initiation of triggered cardiac arrhythmias, notably torsades de pointes.²¹⁻²⁵ Several types of EADs are induced or amplified by fast pacing rates or rate accelerations during bradycardia, just like DADs, and the influence of increased adrenergic stimulation can become proarrhythmic via sudden changes in cellular Ca^{2+} homeostasis.

The importance of EADs and DADs lies in their capacity to provide both the substrate and the trigger for torsades de pointes. It is imperative to better understand the basic mechanisms in situations that are relevant for in-vivo arrhythmia induction, because only this can improve the clinical management of life-threatening ventricular arrhythmias on rational grounds. In this Chapter, we will discuss recent advances in cardiac arrhythmology related to afterdepolarizations from cell to clinic. As a background, we will first review the literature of the 1990s on cellular Ca^{2+} homeostasis and its relation to DADs (sections 2.1 to 2.4). The reader who is familiar with these aspects can proceed with section 2.5 to learn about the new views on Ca^{2+} -dependent mechanisms of EADs and their significance for the initiation and treatment of torsades de pointes.

2. Cellular Mechanisms of Ca^{2+} Homeostasis and Their Relation to Delayed and Early Afterdepolarizations

147

2.1 Ca^{2+} Homeostasis in Normal Cardiac Cells

Ca^{2+} transients underlying excitation-contraction coupling in cardiac cells result mainly from Ca^{2+} release from the SR triggered by Ca^{2+} entry during the action potential. Under normal circumstances, Ca^{2+} entry into cardiac myocytes is carried primarily via I_{CaL} , whereas additional fractions can enter via I_{CaT} and on reverse-mode Na^+ - Ca^{2+} exchange. All three pathways are capable of triggering SR Ca^{2+} release and contraction, but the relative contribution and efficiency is largest for I_{CaL} .²⁶⁻³¹ Additional trigger mechanisms of SR Ca^{2+} release are currently being investigated.³²⁻³⁵ The above-described Ca^{2+} -entry pathways serve two main purposes: (1) they constitute the external trigger for activation of the ryanodine receptors and subsequent release of Ca^{2+} from the SR; and (2) they are largely responsible for cellular Ca^{2+} gain, both at steady state and under circumstances when Ca^{2+} loading is required for an increased contractile performance.

Modern concepts of excitation-contraction coupling rely on a '*local-control theory*', which was originally developed from theoretical models to explain how global

Table 1. – Experimental conditions associated with the generation of delayed (DADs) and early afterdepolarizations (EADs) in monophasic (MAP) and/or transmembrane action-potential (TAP) recordings.

Experimental protocol	DADs	EADs	Frequency dependence	Action-potential duration	References
Altered ionic composition of superfusate [CsCl] _o – MAP	+	+	bradycardia / slow pacing rate (but see also Ref. 191)	increased	151-154
[CsCl] _o – TAP	+	+	slow pacing rate (but see also Ref. 191)	increased	127, 129, 151
[K ⁺] _o -free solution – TAP	+	+	slow pacing rate	increased	126
Cardiac glycosides Digoxin – MAP	+	+	tachycardia / fast pacing rate	decreased	153
Pathophysiological conditions Reoxygenation after anoxia – TAP	+	+	fast pacing rate	increased	192
Repolarization-delaying agents based on slowed inactivation and/or reactivation of <i>I_{Kr}</i> Bay K 8644 – TAP	+	+	fast pacing rate	increased	150

Table 1 – (Continued)

Experimental protocol	DADs	EADs	Frequency dependence	Action-potential duration	References
α_1 - and/or β -Adrenergic receptor stimulation Catecholamines (left stellate ganglion stimulation, epinephrine) - MAP	+	+	fast pacing rate (de-novo EADs or augmentation of EADs upon labile repolarization)	decreased before DADs; increased during EADs	22, 135, 193-195
Catecholamines (norepinephrine, epinephrine) - TAP	+	+	fast pacing rate	decreased before DADs; increased during EADs	76, 196, 197
α -Adrenergic receptor stimulation (phenylephrine, left stellate ganglion stimulation plus propranolol) - MAP	-	+	fast pacing rate (augmentation of EADs upon labile repolarization)	increased	198
α -Adrenergic receptor stimulation (phenylephrine, methoxamine (both plus increased $[CaCl_2]$ or ouabain)) - TAP	+	-	fast pacing rate	increased (but see Ref. 196)	199, 200
β -Adrenergic receptor stimulation (isoproterenol) - MAP	-	+	fast pacing rate (augmentation of EADs upon labile repolarization)	increased	131
β -Adrenergic receptor stimulation (isoproterenol) - TAP	+	+	fast pacing rate	decreased before DADs; increased during EADs	75, 77, 79, 124, 125

Ca^{2+} transients can be graded by the Ca^{2+} influx without uncontrolled regenerative release.³⁶ This theory was supported by morphological studies on the microarchitecture showing a close association between L-type Ca^{2+} channels and ryanodine receptors,³⁷ and subsequently received experimental support from investigations with high-resolution confocal microscopy documenting local functional interaction between these channels.^{38,39} It is now generally thought that cell-wide Ca^{2+} transients result from the spatial and temporal summation of local Ca^{2+} transients, termed ' Ca^{2+} sparks', evoked by relatively high $[\text{Ca}^{2+}]$ near ryanodine receptors upon Ca^{2+} entry through single L-type channels. The occurrence and size of Ca^{2+} sparks are tightly, though not exclusively, linked to Ca^{2+} flux through L-type channels^{38,39} and this fits well with the finding in previous work that the size of global Ca^{2+} transients was regulated by the magnitude of the whole-cell I_{CaL} .^{40,41} In ventricular myocytes, a well-developed t-tubular system promotes homogeneous SR Ca^{2+} release throughout the cell.⁴² The recent observation that Ca^{2+} sparks occur also in cardiac trabeculae under physiological conditions⁴³ indicates that the concepts obtained in studies on single myocytes are applicable to the function of ventricular tissue.

Ca^{2+} release from the SR is controlled by at least three regulators: (1) the size of the triggering Ca^{2+} influx (as mentioned above); (2) the state of the ryanodine receptors; and (3) the Ca^{2+} content within the SR. Recently, Eisner et al⁴⁴ have convincingly argued, both experimentally and theoretically, that the state of the ryanodine receptors as a sole factor will not affect Ca^{2+} release from the SR. Indeed, changes in the gating of the ryanodine receptor and consequently in the size of the Ca^{2+} transient will feedback on transsarcolemmal Ca^{2+} fluxes and this feedback will adjust the SR Ca^{2+} content such that the size of the Ca^{2+} transient returns to its basal level. This process has been called '*autoregulation*' and underscores the importance of the SR Ca^{2+} content. The SR Ca^{2+} content, and thus the amount of Ca^{2+} available for release, depends on: (1) Ca^{2+} uptake into the SR by the Ca^{2+} ATPase; (2) Ca^{2+} efflux (leak); (3) the Ca^{2+} -buffering capacity of the SR; and (4) the $[\text{Ca}^{2+}]_{\text{cyt}}$.⁴⁵ Estimates of the Ca^{2+} content can be made from contractures induced by rapid cooling,⁴⁶ and from caffeine-induced Ca^{2+} release in single cells.⁴⁷ The Ca^{2+} content will affect release not only passively by its effect on the concentration gradient, but also by actively determining the fraction that will be released,⁴⁸⁻⁵⁰ suggesting that there may be a regulatory site for Ca^{2+} on the luminal (intra-SR) side of the ryanodine receptor. As mentioned above, transmembrane Ca^{2+} influx triggers release and serves to fill the SR with Ca^{2+} . Equally important for the net Ca^{2+} flux across the sarcolemma, and thus for the SR Ca^{2+} content, is Ca^{2+} removal from the cell.

$\text{Na}^{+}\text{-Ca}^{2+}$ exchange is the main Ca^{2+} -extrusion mechanism for the beat-to-beat regulation of contraction and relaxation in cardiac myocytes.^{51,52} Because of a stoichiometry of 3 Na^{+} ions : 1 Ca^{2+} ion, inward current is generated when Ca^{2+} is extruded from the cell and outward current is generated when Ca^{2+} enters via this transporter. The direction and magnitude of $\text{Na}^{+}\text{-Ca}^{2+}$ exchange are dependent on the membrane potential and on the intra- and extracellular $[\text{Na}^{+}]$ and $[\text{Ca}^{2+}]$ in the direct vicinity of

the exchanger protein.⁵³⁻⁵⁵ The dynamicity of the action potential and the resultant local Na^+ and Ca^{2+} signals change the reversal potential of the Na^+ - Ca^{2+} exchanger on a continuous basis. Under control conditions, it functions predominantly in the inward mode during the decay phase of the Ca^{2+} transient generating inward current during most of the repolarization of the action potential.^{56,57} To evaluate the importance of inward $I_{\text{Na-Ca}}$ for the course of the action potential, Janvier et al⁵⁸ examined the effects of buffering $[\text{Ca}^{2+}]_{\text{cyt}}$ with BAPTA AM or replacing external Na^+ with Li^+ (both of which inhibit Na^+ - Ca^{2+} exchange) in ferret ventricular myocytes. They found a significant attenuation of the action-potential dome and shortening of the action-potential duration (APD) of 19 and 20% at $\text{APD}_{0\text{mV}}$ and $\text{APD}_{-65\text{mV}}$, respectively, during BAPTA AM, and of 56 and 44% during Li^+ , indicating a major influence of inward $I_{\text{Na-Ca}}$ at baseline.

2.2 Differences of $[\text{Ca}^{2+}]$ Between the Bulk Cytoplasm and the Subsarcolemmal Space

Compelling evidence has accumulated that intracellular concentrations of Na^+ and Ca^{2+} ions can differ considerably between the bulk cytoplasm and the subsarcolemmal space. The discussion on such concentration gradients was instigated in 1990 on the basis of experiments by Leblanc and Hume,³² who showed that reverse-mode Na^+ - Ca^{2+} exchange was capable of triggering Ca^{2+} release from the SR consequent to Na^+ influx through tetrodotoxin-sensitive Na^+ channels. Their data were interpreted as indicative of very high Na^+ concentrations activating reverse Na^+ - Ca^{2+} exchange in a '*functionally restricted intracellular space accessible to Na^+ channels, the Na^+ - Ca^{2+} exchanger, and some of the SR*', which was termed '*fuzzy space*'.⁵⁹ Carmeliet⁶⁰ reviewed the evidence for Na^+ gradients in 1992 and new supportive data have since been published.⁶¹⁻⁶³ Not only Na^+ , but also Ca^{2+} gradients are present in cardiac cells. The fuzzy space is associated with the subsarcolemmal space in the diadic cleft, around the feet of the ryanodine receptors, and is some 10 nm wide. Within this restricted space, and with free diffusion of Ca^{2+} reduced by a factor of 5 to 7, Ca^{2+} fluxes through L-type channels and during Ca^{2+} release create large local gradients.^{64,65} Experimental evidence for these gradients comes mostly from studies examining Ca^{2+} -dependent membrane currents. Given that during voltage clamp $I_{\text{Na-Ca}}$ is a linear function of the intracellular Ca^{2+} concentration,⁶⁶ several groups endeavored to simultaneously measure Ca^{2+} signals with fluorescent Ca^{2+} probes and inward $I_{\text{Na-Ca}}$. Their results confirm that during Ca^{2+} -induced Ca^{2+} release from the SR the Ca^{2+} concentration sensed by the membrane rises and falls more quickly, and is higher than the bulk $[\text{Ca}^{2+}]_{\text{cyt}}$.^{67,68} This hysteresis is further accentuated during cellular Ca^{2+} loading and spontaneous Ca^{2+} release from the SR.^{68,69} Inactivation of L-type Ca^{2+} current during Ca^{2+} release from the SR also exceeds the inactivation expected on the basis of cytoplasmic Ca^{2+} signals.^{70,71} More direct measurements of local Ca^{2+} by membrane-bound Ca^{2+} indicators have been hampered by methodological problems,⁷² although in smooth muscle such gradients have been demonstrated.⁷³

Until now, the importance of functional intracellular Ca^{2+} gradients for the generation of DADs and EADs has received little attention in the literature (but see Trafford et al^{69,74}). Based on the aforementioned data, however, local Ca^{2+} dynamics and resultant activation of $I_{\text{Na-Ca}}$ and other Ca^{2+} -sensitive membrane currents may be of particular relevance. In the discussion on EADs that appear in the setting of Ca^{2+} overload and spontaneous release from the SR (see Figure 1A),⁷⁵⁻⁷⁷ the relative contribution of inward $I_{\text{Na-Ca}}$ to delay repolarization during spontaneous early Ca^{2+} aftertransients may be larger than previously proposed.^{78,79} In the case of slow-rate-dependent EADs (as during treatment with class III antiarrhythmic agents), the close functional coupling of slowly inactivating and/or reactivating I_{CaL} and $\text{Na}^{+}\text{-Ca}^{2+}$ exchange could account for an appreciable inward $I_{\text{Na-Ca}}$ contributing significantly to the primary delay of repolarization (see further section 2.6).

2.3. Cellular Ca^{2+} (Over)load and Spontaneous Ca^{2+} Release from the SR

Spontaneous Ca^{2+} release is usually observed when the Ca^{2+} content of the cells, and of the SR, is very high, a condition often referred to as ' Ca^{2+} overload'. Experimental interventions as diverse as rapid pacing, prolonged depolarization during voltage clamp, high $[\text{Ca}^{2+}]$ or low $[\text{K}^{+}]$ in the superfusate, cardiac glycosides, β -adrenergic receptor stimulation (and many more) produce Ca^{2+} loading via direct or indirect modulation of I_{CaL} or $\text{Na}^{+}\text{-Ca}^{2+}$ exchange. A review of the literature shows that all these interventions have in common that the peak $[\text{Ca}^{2+}]_{\text{cyt}}$ and the amplitude of the Ca^{2+} transient are increased during subsequent action potentials or voltage clamps. Depending on the nature and the intensity of stimulation, the baseline $[\text{Ca}^{2+}]_{\text{cyt}}$ during rest ('diastolic $[\text{Ca}^{2+}]_{\text{cyt}}$ ') may vary from virtually unaltered to significantly upregulated, as indicated in Table 2.

Based on contraction measurements in rat ventricular myocytes during a number of these interventions, Capogrossi et al¹⁷ suggested that the first occurrence of spontaneous SR Ca^{2+} release denotes the state of maximal inotropy in the myocardium. However, in canine ventricular myocytes we found that the twitch potentiation during administration of isoproterenol was not maximal at the first manifestation of aftercontractions, indicating that the amount of SR Ca^{2+} accumulation necessary for spontaneous Ca^{2+} release is lower than that for maximal inotropy.⁷⁷ Species-related differences in SR Ca^{2+} handling may explain these paradoxical findings: in the rat, unlike the dog, the SR works at a near-maximal Ca^{2+} sequestration, which is evident from the frequent spontaneous Ca^{2+} releases at rest.⁸⁰⁻⁸³ This spontaneous SR Ca^{2+} release in rat ventricular myocytes at rest was recently correlated with cellular Ca^{2+} load.⁸¹ In myocytes oscillating at high rates the SR Ca^{2+} content was significantly higher than in quiescent cells. Additionally, an increase in $[\text{Na}^{+}]_{\text{cyt}}$ showed a positive correlation with the frequency of spontaneous Ca^{2+} release, supporting a role for Ca^{2+} loading via modulation of $\text{Na}^{+}\text{-Ca}^{2+}$ exchange.⁸¹ Evidence for the crucial role of SR Ca^{2+} content for spontaneous release also comes from the studies of Ca^{2+} sparks. Cheng et al^{80,84} demonstrated that spontaneous sparks, resulting from the openings of one or a

Table 2. — Diastolic cytoplasmic Ca^{2+} levels during different experimental conditions associated with cellular Ca^{2+} loading (as evidenced by an increased peak $[\text{Ca}^{2+}]_{\text{cyt}}$) or Ca^{2+} overload (as evidenced by the additional occurrence of spontaneous increases of $[\text{Ca}^{2+}]_{\text{cyt}}$).

Experimental condition	Ca^{2+} -indicator	Species and tissue	Diastolic Ca^{2+} level ($[\text{Ca}^{2+}]_{\text{cyt}}$ or ratio) before intervention	Diastolic Ca^{2+} level ($[\text{Ca}^{2+}]_{\text{cyt}}$ or ratio) during Ca^{2+} loading or overload	Relative change of diastolic Ca^{2+} level	References
Altered ionic composition of superfusate [KCl] $_{\text{e}}$ lowered from 4.0 to 0.4 mmol/L, VC	Fura-2	Adult rat ventricular myocytes	75 nmol/L	150 nmol/L	+100 %	18
[KCl] $_{\text{e}}$ lowered from 5.4 to 0.4 mmol/L, FS	Fura-2	Cultured neonatal rat ventricular myocytes	ratio 1.00	ratio 1.30	+30 %	201
[CaCl] $_{\text{e}}$ raised from 1.8 to 3.6 mmol/L, FS	Fura-2	Cultured neonatal rat ventricular myocytes	ratio 0.95	ratio 1.02	+7 %	201
[CaCl] $_{\text{e}}$ raised from 1.8 to 5.4 mmol/L, FS	Fura-2	Cultured neonatal rat ventricular myocytes	ratio 0.95	ratio 1.13	+19 %	201
[CaCl] $_{\text{e}}$ raised from 1.8 to 10.8 mmol/L, spontaneously regularly beating cells	Fura-2	Cultured neonatal rat ventricular myocytes	115 nmol/L	$\approx$ 445 nmol/L	+287 %	202
[NaCl] $_{\text{e}}$ lowered from 130 to 0 mmol/L and [CaCl] $_{\text{e}}$ raised from 1.8 to 5.4 mmol/L, VC	Fura-2	Adult guinea-pig ventricular myocytes	69.6 nmol/L	132.8 nmol/L	+91 %	70
[NaCl] $_{\text{e}}$ lowered from 139 to 74 mmol/L, FS	Fura-2	Cultured neonatal rat ventricular myocytes	ratio 0.95	ratio 1.13	+19 %	201
Cardiac glycosides Dihydroouabain 10^{-6} mol/L, FS	Fura-2	Cultured neonatal rat ventricular myocytes	165 nmol/L	241 nmol/L	+46 %	201
Dihydroouabain 10^{-5} mol/L, FS	Fura-2	Cultured neonatal rat ventricular myocytes	165 nmol/L	289 nmol/L	+75 %	201

Table 2. – (Continued)

Experimental condition	Ca ²⁺ indicator	Species and tissue	Diastolic Ca ²⁺ level ([Ca ²⁺] _{cyt} or ratio) before intervention	Diastolic Ca ²⁺ level ([Ca ²⁺] _{cyt} or ratio) during Ca ²⁺ loading or overload	Relative change of diastolic Ca ²⁺ level	References
<i>Cardiac glycosides (continued)</i>						
Strophantidin 10 µmol/L; resting, unstimulated cells	Fluo-3	Adult guinea-pig ventricular myocytes	92 % *	102 % *	+11 %	203
Strophantidin 100 µmol/L; resting, unstimulated cells	Fluo-3	Adult guinea-pig ventricular myocytes	92 % *	193 % *	+110 %	203
Strophantidin 500 µmol/L; resting, unstimulated cells	Fluo-3	Adult guinea-pig ventricular myocytes	92 % *	361 % *	+292 %	203
<i>α- and/or β-Adrenergic receptor stimulation</i>						
Norepinephrine 10 ⁻⁷ mol/L; VC	Indo-1	Adult rat ventricular myocytes	100 % *	113.8 % *	+14 %	204
Isoproterenol 10 ⁻⁷ mol/L; FS	Fura-2	Cultured neonatal rat ventricular myocytes	165 nmol/L	170 nmol/L	+3 %	201
Isoproterenol 10 ⁻⁶ mol/L; FS	Fura-2	Cultured neonatal rat ventricular myocytes	165 nmol/L	214 nmol/L	+30 %	201
Isoproterenol 10 ⁻⁶ mol/L; VC	Indo-1	Adult rat ventricular myocytes	100 % *	120.0 % *	+20 %	204
Zinterol 10 ⁻⁵ mol/L; VC	Indo-1	Adult rat ventricular myocytes	100 % *	100.2 % *	+0.2 %	204

[Ca²⁺]_{cyt}, ratiometrically determined cytoplasmic Ca²⁺ concentration; VC, whole-cell voltage clamping; FS, field stimulation; ratio, ratio of fluorescent light intensities of excitation- or emission-shifted Ca²⁺ indicator²⁰⁵; *, relative Indo-1 fluorescence level compared to control value before addition of adrenergic agent; *, relative level of corrected Fluo-3 ratio compared to baseline level immediately before solution change.

few SR Ca^{2+} release channels, occurred only at low rates under normal-loading conditions (approximately 0.0001 per s). However, when Ca^{2+} overload was produced by high extracellular $[\text{Ca}^{2+}]$ (10 mmol/L), the rates increased around 4-fold, whereas the sparks' amplitude and size increased 4.1- and 1.7-fold, respectively. Fusion of the local Ca^{2+} transients could set off global Ca^{2+} waves that propagated through the cell by recruiting more Ca^{2+} sparks along the wave front.^{80,84}

The exact mechanisms of spontaneous Ca^{2+} release are still under study. Several groups have now established that for an increase in the Ca^{2+} content of the SR, a larger fraction of the Ca^{2+} pool will be released,⁴⁸⁻⁵⁰ suggestive of a regulation of release by luminal Ca^{2+} . Spontaneous release could result from an increased sensitivity of the release channel to a small increase in diastolic $[\text{Ca}^{2+}]$, either from a small Ca^{2+} influx across the sarcolemma, or due to a passive leak from the SR, perhaps related to saturation of the buffering capacity of the SR. Alternatively, spontaneous release could be unrelated to the normal calcium-induced calcium release mechanism, as suggested by Fabiato,⁸⁵ and perhaps be due to altered properties of the ryanodine receptor.

Independent of the underlying mechanism, Ca^{2+} imaging in cardiac myocytes has revealed two patterns of spontaneous Ca^{2+} release: (1) focal Ca^{2+} release with no or limited propagation along the SR; and (2) Ca^{2+} release propagating as a wave through the entire cell (eg, see References 80, 84, 86 to 90). Numerous groups have investigated the interplay between these types of Ca^{2+} release and arrhythmogenic I_{T1} and DADs (eg, see References 18, 74-76, 79, 91-95). For focal release and Ca^{2+} waves, however, quantitative interpretation of such data remains difficult since membrane currents always represent a spatial average.

2.4 Ionic Mechanisms of DADs

It is generally accepted that DADs are caused by I_{T1} evoked by spontaneous Ca^{2+} release in the setting of cellular Ca^{2+} overload. This sudden increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ modulates Ca^{2+} -sensitive membrane currents via channel- or transporter-specific mechanisms. Three different ionic currents, alone or in combination, have been inferred in the generation of I_{T1} since its first description in 1976.⁹⁶ These are $\text{I}_{\text{Na-Ca}}$,^{74,93,95,97-102} non-selective cation current (I_{NS}),^{97,103-106} and Ca^{2+} -activated Cl^- current ($\text{I}_{\text{Cl}(\text{Ca})}$).^{74,93,101,102}

Kass et al⁹⁷ were among the first to speculate on the ionic basis of I_{T1} and proposed that this current, induced by strophanthidin in calf cardiac Purkinje fibers, might be carried by a 'leak channel' or that it reflected ' Ca^{2+} extrusion by an electrogenic Ca-Na exchange'. The contribution of $\text{I}_{\text{Na-Ca}}$ to I_{T1} has been estimated by comparing the current-voltage relationships of the two and by determining the characteristics of $\text{I}_{\text{Na-Ca}}$ and I_{T1} during spontaneous Ca^{2+} release from the SR. With respect to the latter, Beuckelmann and Wier⁵⁴ showed that the reversal potential of the Ni^{2+} -sensitive $\text{I}_{\text{Na-Ca}}$ in guinea-pig ventricular myocytes increased linearly with the log $[\text{Ca}^{2+}]_{\text{cyt}}$ (see also sections 2.1. and 2.2.). The high $[\text{Ca}^{2+}]_{\text{cyt}}$ during spontaneous Ca^{2+} release would thus shift the reversal potential of $\text{I}_{\text{Na-Ca}}$ towards very positive membrane potentials causing

the current to be inward at voltages relevant for the action potential. Lipp and Pott⁹⁹ examined the direction of the I_{T1} during voltage clamps between -90 and +75 mV, and found it to remain inward over the whole range. They concluded that I_{Na-Ca} was the major charge-carrying mechanism for I_{T1} . This conclusion was also drawn by Benndorf et al¹⁰⁰ after similar experiments on Ca^{2+} -overloaded guinea-pig ventricular myocytes. Other groups investigated I_{T1} in the assumed absence of I_{Na-Ca} . Sipido et al⁹² could not find evidence for a significant contribution of I_{NS} to the current oscillations during spontaneous SR Ca^{2+} release in guinea-pig ventricular cells in such condition. In sheep cardiac Purkinje fibers, when the extracellular $[Na^+]$ was completely replaced with isotonic $CaCl_2$, the remaining I_{T1} had a reversal potential of -37 mV suggesting a channel mechanism as the charge carrier.¹⁰³ Giles and Shimoni¹⁰⁵ induced I_{T1} in single rabbit ventricular myocytes by the application of low- $[K^+]$ superfusate or strophanthidin, and mentioned the possibility of I_{NS} as an alternative explanation for I_{T1} . Verkerk et al¹⁰¹ wondered whether the controversial findings on I_{NS} could be attributable to species- or cell-type-related differences and compared the I_{T1} characteristics of Purkinje and working myocytes in sheep hearts. In neither cell type an appreciable magnitude of I_{NS} could be measured.¹⁰¹ Also in rabbit⁹³ and canine ventricular myocytes¹⁰² the absence of I_{NS} in I_{T1} was reported. In summary, all experiments mentioned so far support the role of the Na^+-Ca^{2+} exchange in I_{T1} , but the exact contribution of I_{NS} remains controversial.

A third relevant current is $I_{Cl(Ca)}$, which may have been present as I_{NS} in some of the previously mentioned studies. $I_{Cl(Ca)}$ requires SR Ca^{2+} release to become activated, but its time course does not follow the time course of the $[Ca^{2+}]_{cyt}$ as measured with indo-1.¹⁰⁷ Zygmunt argued that the rapid decline of this anion current could not be explained by Ca^{2+} -dependent inactivation.¹⁰⁸ Rather, the transient nature could follow $[Ca^{2+}]$ changes in the subsarcolemmal compartment.^{69,107} $I_{Cl(Ca)}$ makes up a substantial portion of I_{T1} in the dog: quantitative estimates in left ventricular (LV) midmyocardial (M-)cells indicate that it accounts for up to 40% of I_{T1} during Ca^{2+} overload, whereas I_{Na-Ca} represents the additional 60%.¹⁰² In ferret,⁷⁴ rabbit,^{93,106,109} and sheep¹⁰¹ $I_{Cl(Ca)}$ has also been reported. However, in guinea-pig⁹² and human myocytes⁹⁵ the current was not observed.

Depending on the induction protocol, DADs can appear at short or long coupling intervals after the action potential. Typical examples of short-coupled DADs are those induced by cardiac glycosides or $[K^+]_o$ -depleted superfusates due to inhibition of the Na^+-K^+ pump. They occur immediately after the action potential (often before repolarization is complete) and can exhibit an oscillatory pattern with decreasing amplitudes during diastole. These membrane oscillations reflect repetitive spontaneous Ca^{2+} releases from the SR under circumstances when both $[Na^+]_{cyt}$ and $[Ca^{2+}]_{cyt}$ are high with opposite effects on inward I_{Na-Ca} (Table 2). In the example of arrhythmogenic β -adrenergic receptor stimulation the situation is different. Although Ca^{2+} influx during the action potential is greatly enhanced, the diastolic $[Ca^{2+}]_{cyt}$ is maintained at relatively low levels due to an increased Ca^{2+} uptake by the SRATPase (Table 2). In this

case, the $[\text{Na}^+]_{\text{cyt}}$ may be decreased. The DADs have a longer coupling interval reflecting the time needed by the SR to become overloaded.

In various pathophysiological conditions, alterations of Ca^{2+} homeostasis can predispose to an increased occurrence of DADs. In the dog with chronic complete atrioventricular block (AVB; see Chapter 5), the upregulation of $\text{Na}^+-\text{Ca}^{2+}$ exchange and increased Ca^{2+} release from the SR are associated with an enhanced propensity to Ca^{2+} overload and spontaneous SR Ca^{2+} release. In-vivo experiments in chronic AVB, but not in acute AVB, demonstrate the induction of DADs and triggered ectopic beats following short pacing trains. This arrhythmic activity is related to a critical increase of the LV pressure rise, and can be enhanced by ouabain and suppressed by ryanodine and lidocaine.

In conclusion, the ionic mechanisms of DAD can vary according to cell type, species and experimental condition or functional state, but cellular Ca^{2+} overload is the common substrate causing spontaneous Ca^{2+} release from the SR during diastole. This primary event activates Ca^{2+} extrusion via the $\text{Na}^+-\text{Ca}^{2+}$ exchanger at least to some degree. Although beneficial by itself, inward $I_{\text{Na-Ca}}$ can trigger premature impulses and arrhythmias.

2.5 Ionic mechanisms of EADs

The appearance and mechanisms of EADs are diverse as they occur in various conditions. EADs present as transient retardations or reversals of the repolarization during phases 2 and 3 of the action potential, often in the setting of action-potential prolongation. Although the term '*early afterdepolarization*' per se would not comprise a mere retardation of the repolarization, this is actually the priming event of the EAD formation, and must be incorporated into any theoretical mechanistic framework on EADs. At the ionic level, a decrease in outward current(s), an increase in inward current(s), or combinations of the two can unbalance the repolarization such that EADs arise. However, due to the very complex dynamics and overlapping of transsarcolemmal ionic fluxes during the action potential, the impact of individual current alterations on repolarization characteristics cannot easily be estimated. In the case of the congenital long-QT syndromes, one can pinpoint single current defects as the primary event.¹¹⁰ However, in most conditions a more complex situation exists. Experimental electrophysiological studies on EADs have often relied on pharmacological agents and slow pacing modes to cause the initial action-potential prolongation via reductions of I_{Kr} ,¹¹¹⁻¹¹⁴ or slowed inactivation of I_{Na} ¹¹⁴⁻¹¹⁷ and I_{CaL} .^{118,119} In many of these previous works, the major emphasis was on the currents generating the actual upstroke of the EAD and convincing arguments were found for the role of reactivating I_{Na} and I_{CaL} (dependent on the actual experiment). This was reviewed in a number of articles.^{19,20,120} The significance of I_{CaL} for EADs is due to its voltage-dependent activation falling in the range of membrane potentials (-35 to 0 mV) where repolarization is delayed and EADs arise. Based on experiments with Bay K 8644, January and coworkers^{118,119,121,122} concluded that the induction of EADs requires: '(i) a condi-

tioning phase controlled by the sum of membrane currents present near the action potential plateau and characterized by lengthening and flattening of the plateau within a voltage range where, (ii) recovery from inactivation and reactivation of L-type Ca^{2+} current to carry the depolarizing charge can occur'.¹¹⁹ In the presence of pharmacological agents (eg, Bay K 8644 or ATX-II) or channel modifications that slow inactivation of I_{CaL} or I_{Na} , these currents are obviously of prime importance. However, in the absence of such conditions, inward $\text{I}_{\text{Na-Ca}}$ is likely to play a significant role in the initial delay in repolarization designated 'conditioning phase'¹¹⁹ or 'conditional phase'.^{77,78} The conditional phase appears as the transient delay in repolarization that can, but does not have to, be followed by an EAD upstroke or triggered action potential. Based on our own work and accumulating evidence in the literature, a primary role for $\text{Na}^+\text{-Ca}^{2+}$ exchange is not only restricted to the conditional phase of EADs related to Ca^{2+} overload and spontaneous Ca^{2+} release from the SR, but applies also to EADs that occur in the setting of less extreme Ca^{2+} loading. This is explained in the next section.

2.6 Evidence for $[\text{Ca}^{2+}]_{\text{cyt}}$ -Dependent Mechanisms of EADs

There are three major observations supporting the contribution of $[\text{Ca}^{2+}]_{\text{cyt}}$ -dependent mechanisms to EADs. First, dependent on the mode of induction, DADs and EADs can appear jointly in consecutive action potentials or even in relation to the same action potential (Table 1). A second argument for $[\text{Ca}^{2+}]_{\text{cyt}}$ -dependent mechanisms derives from the observation that several types of EADs are induced or amplified by fast pacing rates or rate acceleration during bradycardia, just like DADs. Also, the influence of increased adrenergic stimulation can induce EADs via sudden changes of cellular Ca^{2+} homeostasis. Clinically, increased adrenergic tone and sudden rate accelerations are often involved in the initiation of torsades de pointes. A third argument is that experimental interventions known to attenuate $\text{Na}^+\text{-Ca}^{2+}$ exchange can also inhibit EADs under certain conditions. These three arguments can be found in a number of examples on $[\text{Ca}^{2+}]_{\text{cyt}}$ -dependent EADs described below and illustrated in the Figures 1 to 3.

First, we analyze the EADs induced by isoproterenol. During intense β -adrenergic receptor stimulation, arrhythmogenic responses in ventricular myocytes are accompanied by spontaneous Ca^{2+} release from the SR during both diastole and systole (Figure 1A), often in conjunction with the same action potential.^{75,76,123} The systolic spontaneous releases appear as early Ca^{2+} aftertransients following the (twitch-related) I_{CaL} -induced Ca^{2+} release and they have mechanical corollaries: early aftercontractions.^{77,124} We have presented compelling evidence that the conditional phase of EADs induced by isoproterenol is generated by inward $\text{I}_{\text{Na-Ca}}$ activated by the early Ca^{2+} aftertransients. Thus, these EADs depend on a SR-dependent mechanism for their rise,⁷⁷ as also suggested earlier.¹²⁵ It is noteworthy to mention that the simulation study of Zeng and Rudy⁷⁸ on isoproterenol-induced EADs did not incorporate a secondary systolic rise of $[\text{Ca}^{2+}]_{\text{cyt}}$ and for that reason must have underestimated the contribution of inward $\text{I}_{\text{Na-Ca}}$. We observed that DADs and EADs induced by isoproterenol

Figure 1 (page 160). – $[\text{Ca}^{2+}]_{\text{cyt}}$ -dependent mechanisms of EADs in vitro.

A, Spontaneous Ca^{2+} release from the SR underlying both EAD and DAD activity in a canine ventricular myocyte treated with isoproterenol. EADs and DADs appear in relation to the same action potentials during pacing at a cycle length of 1000 ms. Arrows indicate the termination of pacing. These are whole-cell voltage recordings with the fluorescent Ca^{2+} indicator indo-1. The calibration bars represent 1 fluorescence ratio unit for $[\text{Ca}^{2+}]_{\text{cyt}}$ activity, 50 mV for whole-cell voltage traces, and 2 s for the time scale. Reproduced from Reference 75. **B**, Late EADs arising from membrane potentials more negative than -35 mV during administration of epinephrine in a clofilium-pretreated canine Purkinje fiber. In the upper panel, the action potential at baseline is depicted. In the middle panel, action-potential prolongation by clofilium is shown. No EADs or DADs are present. In the lower panel, upon the additional administration of epinephrine at low concentration, pronounced formation of EADs is present at cycle lengths greater than 1000 ms. In these recordings the cycle length is 2000 ms. Reproduced from Reference 140. **C**, Acceleration-induced EADs. In the left panel, action potentials are recorded from canine LV M-tissue, treated with the I_{Kr} blocker E-4031, during a sudden increase of the rate from a cycle length of 2000 to 1000 ms. The transient appearance of EADs is accompanied by action-potential prolongation. In the right panel, the action potentials are superimposed, showing the last beat at the cycle length of 2000 ms, and some of the first nine action potentials at 1000 ms. Reproduced from Reference 143.

Figure 2 (page 161). – In-vivo appearance of EADs and DADs in anesthetized dogs with chronic AVB.

In all three panels, the ECG lead II and a LV endocardial MAP recording are shown. **A**, DAD (closed asterisk), EADs (open asterisks), and triggered activity in consecutive MAPs after a pacing train of 8 stimuli with an interstimulus interval of 300 ms under baseline conditions. Paced beats are indicated by S. Just before pacing the idioventricular cycle length is 1360 ms, increasing to 1990 ms thereafter. The first beat postpacing shows EAD formation and a triggered action potential on the MAP signal. **B**, Induction of phase-2 and phase-3 (late) EADs in consecutive beats during administration of the class III antiarrhythmic agent ibutilide. The occurrence of EADs follows a MAP prolongation from 375 ms at baseline (PRE-DRUG) to 555 ms during ibutilide and is related to the triggering of multiple ectopic beats (as shown on the right). **C**, Acceleration-induced accentuation of EADs during a sudden increase in the pacing rate from a cycle length of 1500 to 1000 ms. The emergence of EAD upstrokes is a transient phenomenon upon the rate change, which fades away within 1 minute thereafter.

were completely suppressed by the cation Ni^{2+} , whereas their accompanying delayed and early aftercontractions remained present.⁷⁷ This strongly suggested the contribution of inward $\text{I}_{\text{Na-Ca}}$ to the generation of both types of afterdepolarizations. Priori and Corr¹²⁵ noted similar suppressive actions on EADs by perfusion with low- $[\text{Na}^+]_o$ solution or treatment with the (aspecific) Na^+ - Ca^{2+} -exchange blocker benzamil. Using fluorescent Ca^{2+} imaging, two groups^{79,91,126} observed that the spatial features of Ca^{2+} transients associated with afterdepolarizations were different for DADs (heterogeneous pattern indicating focal, spontaneous SR Ca^{2+} release) *versus* EADs (homogeneous pattern suggesting I_{CaL} -induced Ca^{2+} release). This is in line with the concept of different ionic mechanisms underlying DADs and EADs. However, the same groups also emphasized that EADs can consist of different types with different etiology; Miura et al¹²⁶ showed that late EADs induced during perfusion with $[\text{K}^+]_o$ -free solution were associated with the heterogeneous $[\text{Ca}^{2+}]_{\text{cyt}}$ pattern akin to DADs, but much different from the homogeneous pattern accompanying EADs earlier in repolarization; and De

IN VITRO

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Ferrari and coworkers mentioned preliminary data on isoproterenol-induced EADs with similar characteristics.⁷⁹

Next, we analyze the so-called '*late EADs*' initiating at membrane potentials more negative than the range at which I_{CaL} can be activated.¹²⁷⁻¹²⁹ Although conceptually I_{Na} , I_{CaT} or I_{NS} could underlie these late EADs, there is increasing evidence that inward I_{Na-Ca} has the primary role. It has been shown that interventions causing cellular Ca^{2+} loading augment these EADs.¹²⁸⁻¹³⁰ Such an effect is produced by the application of Cs^+ , which inhibits K^+ currents, and epinephrine. In Purkinje fibers or ventricular myocytes, relatively low concentrations of these agents do not generate EADs when applied separately. The explanation for late-EAD generation under these conditions is in increasing the APD by Cs^+ and increasing Ca^{2+} sequestration and release from the SR by epinephrine at the same time. Inward I_{Na-Ca} is the main current responsible for the initiation of the afterdepolarizations as demonstrated by the inhibitory effects of high extracellular $[Ca^{2+}]$ and of ouabain (the latter being due to the effect of increased $[Na^+]_{cyt}$ on the exchanger).

Finally, we focus on the conditional phase of EADs induced during congenital or acquired inhibition of I_{Kr} and/or I_{Ks} . We recall that the typical example of the congenital form of inhibition is the long-QT syndrome, whereas acquired forms are seen, for example, during treatment with class IA or class III antiarrhythmic agents. In the clinic, these syndromes are associated with an increased regional dispersion of ventricular repolarization, generation of EADs, premature ventricular beats and a serious risk of torsades de pointes (eg, see References 22, 131-135 and 132, 134, 136-139). Most often, torsades de pointes is precipitated by sudden accelerations or short-long-short sequences of the heart rate during bradycardia,^{21,23-25} whether or not under the influence of an increased sympathetic tone.²² In an attempt to comprehend the behavior and proarrhythmic potential of abnormal ventricular repolarization and EADs under these circumstances, and to appreciate the significance of inward I_{Na-Ca} herein, we must return to the cellular level. The influences of increased sympathetic tone, rate accelerations and short-long-short sequences are consecutively addressed.

Patterson et al¹⁴⁰ evaluated the electrophysiological actions of the class III antiarrhythmics *d,l*-sotalol and clofilium in canine cardiac Purkinje fibers and found that action-potential prolongation by these agents is necessary but not sufficient to produce afterdepolarizations and triggered activity. The elicitation of slow-rate-dependent EADs was only observed after the additional administration of epinephrine at low concentrations (10-100 nmol/L; Figure 1B). In the case of clofilium, fast-rate-dependent DADs were also seen. Epinephrine alone did not produce afterdepolarizations. The authors tested whether inward I_{Na-Ca} could contribute to the generation of EADs and found good arguments that this is indeed so: a temporary increase of the extracellular $[Ca^{2+}]$ to 5 mmol/L, which attenuates inward I_{Na-Ca} ,⁵⁵ suppressed EAD formation.¹⁴⁰ The same group more directly evaluated the role of Na^+-Ca^{2+} exchange in class III EAD.¹⁴¹ During arrhythmogenic actions of clofilium and *d,l*-sotalol in canine Purkinje fibers and subepicardial ventricular myocytes, inhibition and subse-

Figure 3. – Pacing-dependent torsades de pointes in the presence of *d*-sotalol (2 mg/kg/5 min) in an anesthetized dog with chronic AVB.

Shown are the ECG leads I, II and III, and a LV endocardial MAP recording. Just before pacing the idioventricular cycle length is 2270 ms and the QT interval measures 500 ms. The pacing protocol consists of 8 stimuli with 600-ms intervals, 1 stimulus at 1200 ms and a short-coupled extra beat (8×600 / 1200 / EXTRA; indicated by S). Pacing-induced phase-2 and phase-3 EADs, and related triggered ectopic beats precipitate torsades de pointes. In this case, the arrhythmia is self-terminating. Reproduced from Reference 190.

quent transient stimulation of inward I_{Na-Ca} was obtained by substituting LiCl for NaCl, followed by the return to normal extracellular $[Na^+]$. Whereas EADs had been absent at baseline (class III agents only), this forth-and-back change of solutions potentiated EAD formation in a significant number of preparations upon reintroduction of the normal $[Na^+]$ with EAD take-off potentials being more negative than -60 mV.

Proarrhythmic effects of sudden rate acceleration have been related to the marked electrical characteristics of M cells in the midmyocardial layer of the ventricle. Sicouri et al¹⁴² found that rate acceleration in the presence of *d*-sotalol led to a paradoxical transient prolongation of the action potential and the occurrence of EADs in LV M-cells, but not in endo- or epicardial cells. This behavior caused a temporary increase in the transmural dispersion. Burashnikov and Antzelevitch¹⁴³ investigated the influence of rate accelerations on EADs induced by the I_{Kr} blocker E-4031 in tissue preparations from the canine LV wall and Purkinje fibers. Sudden accelerations from initially slow pacing rates caused transient EAD activity if none existed before or resulted in augmentation of preexisting EADs in the majority of M-tissues (Figure 1C) and to a lesser degree in Purkinje fibers. A large transmural dispersion of repolarization resulted from the absence of such effect in the endo- and epicardial layers. The investigators applied ryanodine, flunarizine and low extracellular $[Na^+]$ to examine whether

cellular Ca^{2+} loading and increased activity of the Na^+ - Ca^{2+} exchanger could be mechanistically involved. In support of such a role, all three interventions abolished the acceleration-induced EADs and the APD prolongation.¹⁴³ With regard to the cellular correlates of the in-vivo short-long-short sequence, the following: Burashnikov and Antzelevitch¹⁴³ reported on the ability of single premature ventricular beats to induce EADs and action-potential prolongation in M-cells. In line with an earlier study,¹⁴⁴ this potentiating effect could be explained by increased Ca^{2+} transients accompanying the post-extrasystolic beats, which fitted well with a central role for the Na^+ - Ca^{2+} exchanger. Similar interpretations were made by Viswanathan and Rudy¹⁴⁵ for pause-induced EADs in a simulation study on the initiation of torsades de pointes. Single pauses interrupting the steady-state pacing rates caused prolongation of the post-pause action potential due to a smaller I_{Ks} (more deactivation) and an enhanced $I_{\text{Na-Ca}}$ (larger post-pause Ca^{2+} transient). This delayed repolarization set the stage for reactivation of I_{CaL} to depolarize the membrane and generate the EAD upstroke.¹⁴⁵

3. Afterdepolarizations and Arrhythmogenesis in Myocardial Tissue and the Intact Animal Heart

In this part of the review, we will focus on triggered activity in multicellular preparations and the intact animal heart. Because we will concentrate on the literature of the 1990s, the emphasis will be on direct evidence (microelectrodes or MAP catheters) concerning: (1) the occurrence and propagation of acquired EADs, and their consequences for arrhythmogenesis; (2) circumstances when EADs and DADs occur in the same experiment; (3) other circumstances when EADs appear to be linked to myocardial Ca^{2+} overload.

For an excellent recent review on the initiation and propagation of DADs in myocardial tissue we refer to ter Keurs et al.¹⁴⁶

3.1 EADs in Multicellular Preparations: Consequences for Arrhythmogenesis

In Purkinje-muscle preparations,^{20,112,147} a number of studies demonstrated more pronounced changes of the APD in response to either bradycardia and/or repolarization-delaying drugs in Purkinje fibers than in ventricular muscle. Under these specific circumstances, a marked dispersion of repolarization is often observed and is an important determinant of arrhythmogenesis. Furthermore, there is an increased incidence of EADs in Purkinje fibers, which can give rise to propagated ectopic beats on the basis of: (1) direct triggering of EADs in Purkinje fibers (Figure 1B), most likely from phase-3 EADs; or (2) prolonged repolarization-dependent reexcitation. The capacity for reexcitation will depend on the difference in membrane potential between connected cells, but also on the excitability threshold of the already-repolarized cells. Using canine LV-wedge preparations, Antzelevitch and coworkers^{113,114,148,149} found similar results with now the M-layer as the culprit cell

type. The APD and EAD sensitivity of M-tissue for bradycardia and/or class III agents was significantly higher than of the surrounding endo- and epicardium. Similar observations were made for the occurrence of DADs during treatment with acetylcholine and Bay K 8644.¹⁵⁰ It has to be stated that, by no means, M-cells are the electrophysiological or pharmacological equivalent of Purkinje fibers, but their responses to bradycardia and certain drugs are quite alike. Microelectrode recordings in LV-wedge preparations showed that transmural dispersion (developing through nonhomogeneous responses of the M-layers to chromanol 293B *plus* isoproterenol,¹⁴⁹ *d*-sotalol,¹¹⁴ and ATX-II¹¹⁴) was the prerequisite for spontaneous or pacing-induced polymorphic arrhythmias, mimicking torsades de pointes in the intact heart. The initiating beats of the spontaneous arrhythmias most frequently seemed to originate from the deep subendocardium (either in Purkinje or in M-cells).

3.2 Simultaneous Appearance of DADs and EADs in the Intact Heart

To the best of our knowledge, Patterson and coworkers¹⁵¹ were the first to describe that EADs and DADs could appear together in the same intact heart, albeit at different time points after the administration of intravenous CsCl. At first instance (2 to 5 min after the start of infusion), Cs⁺ caused action-potential prolongation with the appearance of EADs. Thereafter, the MAP duration started to decrease and at 8 to 10 min DADs appeared. Surprisingly, the coupling interval of the afterdepolarizations remained similar, indicating that depending on the APD an EAD (long APD) or a DAD (short APD) could occur. It was also the Oklahoma-City group that suggested that these EADs and DADs could be based on similar cellular mechanisms via alterations of myocardial Ca²⁺ homeostasis.¹⁵¹ Since 1990, more publications have indicated the simultaneous appearance and common etiology of certain EADs and DADs in the intact heart.^{152,153} Using floating microelectrodes in cats, Xie and Xie¹⁵⁴ again reported the occurrence of EADs and DADs at different time points after administration of CsCl. Most EADs arose during phase 3 of the transmembrane and monophasic action potential. These investigations provided evidence that the afterdepolarizations recorded in transmembrane action potentials could be visualized simultaneously by MAP recordings. In a study by Xu et al.,¹⁵³ it was shown in guinea pigs with the use of MAP recordings from the midmyocardium that digoxin induced DADs and phase-3 EADs, which were both suppressed by verapamil. Some types of EADs converted to DADs in the MAP after administration of the K⁺-channel opener pinacidil, which shortens the APD. These authors concluded that '*late phase 3-EADs generated under conditions of Ca²⁺ overload and DADs share similar properties*'.¹⁵³

We have described that chronic AVB in the dog results in: (1) electrical remodeling (nonhomogeneous action-potential prolongation); (2) an enhanced contractile performance at the slow idioventricular heart rate; and (3) biventricular hypertrophy.¹⁵⁵ In some of these (anesthetized) dogs, short pacing trains can induce both EADs and DADs, and related ectopic beats under baseline conditions. A typical example is shown in Figure 2A. Ryanodine abolishes both these EADs and DADs (not

shown), suggesting a common cellular mechanism for the two. In experiments with class III antiarrhythmic drugs at clinically relevant doses, the dogs show the appearance of EADs in MAP recordings. As illustrated in Figure 2B, these EADs can occur at the end of the plateau (phase 2) and during phase 3 of the repolarization, often in consecutive beats. More importantly, EADs and related ectopic beats can be induced by fast-pacing modes leading to the induction of torsades de pointes (Figure 3). In correspondence with the in-vitro experiments by Burashnikov and Antzelevitch,¹⁴³ we further find that sudden increases in pacing rate (eg, from a cycle length of 1500 to 1000 ms) cause the emergence or accentuation of EADs, which are transient usually restricted to a period of seconds to 1 minute of the new rate (Figure 2C). These observations are not in contrast with the known APD adaptation to heart-rate increases during pacing or adrenergic activation, but rather uncover the delicate balance of ventricular repolarization in this animal model, which needs time to adapt to a new steady state.

3.3 EADs Occurring in Conditions Associated with Myocardial Ca^{2+} Overload

In the dog with chronic AVB and acquired QT prolongation, a relation between myocardial Ca^{2+} overload and the generation of class III-dependent EADs and torsades de pointes has been suggested by the attenuating (EADs) and preventive (pacing-induced torsades de pointes) effects of the agents ryanodine and flunarizine.¹⁵⁶ Similarly, in a rabbit model of torsades de pointes (co-administration of methoxamine and almokalant), Carlsson et al¹⁵⁷ showed the prevention of spontaneous torsades de pointes when the rabbits were pretreated with nisoldipine and flunarizine.

4. Clinical Management of Arrhythmias in the Congenital and Acquired Long-QT Syndromes

Early electrophysiological studies with recordings of endocardial electrograms and MAPs in patients with congenital and acquired long-QT syndromes implicated EADs as contributors to the T-U waves and generators of arrhythmias.¹⁵⁸⁻¹⁶¹ Later investigations with MAP recordings in humans indicated the appearance of EADs that were enhanced at longer cycle lengths and with adrenergic stimulation, and were diminished by β -adrenergic blockers, Ca^{2+} -channel blockers, and K^{+} -channel openers.^{22,131,133,135,137-139,162-167} Because EADs are related to the congenital and acquired long-QT syndromes, the recording of these deflections in patients is of help in the understanding of: (1) the pathogenesis; (2) the influence of EADs on the surface ECG; and (3) the effects of treatment. The inciting effects of emotion and exercise in the congenital long-QT syndrome were first recognized in the report of Jervell and Lange-Nielsen,¹⁶⁸ and have been reiterated in subsequent reports.¹⁶⁹ Based on these observations, β -adrenergic blockade and left cardiac sympathectomy were empirically derived successful therapies.¹⁷⁰⁻¹⁷²

The acquired long-QT syndromes are characteristically seen during bradycardia with the surface ECG showing accentuated T- and U-wave abnormalities. Irregularities in the rhythm, such as premature beats, as caused by ventricular ectopy or atrial fibrillation, can lead to the induction of torsades de pointes. Most drugs prolonging ventricular repolarization may cause torsades de pointes, but the arrhythmia is usually seen in a typical clinical context, which includes female preponderance, bradycardia, irregular rhythm, and hypopotassemia and/or –magnesemia. The plasma level of the repolarization-prolonging drug is often not elevated, which suggests an idiosyncratic rather than a toxic effect. In some patients with a (presumed) acquired long-QT syndrome, *de-novo* gene mutations can be identified, which suggest the presence of a forme fruste of the congenital long-QT syndrome.¹⁷³ Clinical studies on the electrophysiological characteristics of the acquired long-QT syndrome have been conducted mostly in forms induced by drugs, such as quinidine,¹³⁷ procainamide,¹³⁸ disopyramide,¹³⁹ and the novel class III drug almokalant.¹⁷⁴ The provocation of arrhythmias by long cycle lengths was emphasized in the acquired long-QT syndromes¹⁶⁹ and it is operative also in the congenital long-QT syndromes. During these interventions the behavior of the repolarization phase was often dynamic, with T-U waves showing abnormal configurations such as bifidity, negativity, and 2 : 1 alternation.¹⁷⁴ The U-wave amplitude correlated strongly with the EAD amplitude, especially when the latter was recorded from a corresponding endocardial site (eg, ECG lead V₂ *versus* high right-ventricular MAP).¹³⁹

A sustained increase of the heart rate is an effective maneuver to suppress torsades de pointes acutely in the acquired long-QT syndromes,¹⁶⁹ and isoproterenol administration and permanent pacemakers have been effective in the congenital long-QT syndromes.¹⁷⁵ Ca²⁺-channel blockers, including intravenous Mg²⁺, have been reported to be effective in suppressing arrhythmias.^{22,169} The correspondence of these clinical observations with observations of the responses of EADs in MAP recordings supports the hypothesis that EADs are a basic trigger mechanism for arrhythmogenesis in the long-QT syndromes.

The therapeutic benefit of β -adrenergic blockade, left cardiac sympathectomy, and Ca²⁺-channel blockers implicates Ca²⁺ entry and Ca²⁺ loading in the triggering mechanism, but does not allow the identification of I_{CaL} or I_{Na-Ca} as the major current generator for EADs. Reduction of I_{CaL} by these therapeutic measures would also result in reduction of Ca²⁺ loading and Na⁺-Ca²⁺ exchange, and inhibit spontaneous SR Ca²⁺ release. In all reports of EADs shown in MAPs in both congenital and acquired long-QT syndromes, these membrane responses occurred during phase 3 of the action potential. In some studies EADs also appeared during phase 2, but when triggering was observed, it was observed to be timed with phase 3.^{138,162,163} While MAPs do not allow precise determination of the membrane potential at which the EADs originate, the relative location on phase 3 strongly suggests an initial potential that is often more negative than –35 mV, excluding a role for I_{CaL} in those cases. These observations implicate I_{Na-Ca} rather than I_{CaL} as a common current generator for EADs and triggering.

In the congenital long-QT syndromes, strategies to abbreviate repolarization and the QT interval, thereby eliminating the basis for EAD generation, are appropriate. Recent discoveries of gene mutations and their functional consequences have presented new therapeutic options. In LQT3, various mutations of the SCN5A gene result in abnormal inactivation of the Na^+ channel and an excess of persistent Na^+ current that prolongs the plateau of repolarization.¹⁷⁶ This abnormality is corrected by Na^+ -channel blocking agents of the IB class. Mexiletine has been shown to abbreviate the QT interval to normal or near normal in patients with this defect.¹⁷⁷ However, the efficacy of class IB agents in the prevention of sudden death has not yet been demonstrated.

In LQT2, a defect in HERG causes diminished I_{Kr} and delayed repolarization.¹⁷⁸ I_{Kr} is enhanced by increased extracellular K^+ .¹⁷⁹ It has been shown that intravenous infusion of K^+ normalizes the QT in LQT2.¹⁸⁰ The effects of long-term measures to elevate serum $[\text{K}^+]$ are still poorly explored. Recently, Tan et al¹⁸¹ presented a female patient with LQT2, who had been treated with K^+ -sparing drugs and increased K^+ intake to normalize her ECG and prevent torsades de pointes. The authors found that in the presence of a normal renal function, K^+ homeostasis limited a long-lasting rise of serum $[\text{K}^+]$.¹⁸¹ Enhancement of I_{Kr} or reduction of persistent I_{Na} would tend to abbreviate repolarization not just in the defects specifically involving these currents, but the effect predictably might be less if the intervention were applied when other currents were responsible for the prolongation of repolarization. In a report comparing the effects of mexiletine in patients with LQT1, LQT2 and LQT3, the effect appeared to be greatest in LQT1, but there was a trend towards reduction in the QT interval in both LQT2 and LQT3.¹⁷⁷

In the congenital long-QT syndromes, the commonly used therapies, β -adrenergic blockade, left cardiac sympathectomy, and pacing, developed empirically as measures for suppressing arrhythmias rather than normalizing repolarization. Because I_{Ks} is enhanced by β -adrenergic receptor stimulation, the deficiency of I_{Ks} would impair action-potential shortening during adrenergic stimulation and exaggerate the disproportionately long duration of repolarization at adrenergically-induced higher heart rates. There is an indication supported by preliminary observations that adrenergic triggers may be most potent in mutations causing deficient I_{Ks} : LQT1, LQT5, and Jervell and Lange-Nielsen syndrome.^{182,183} LQT1 is the most common form of the syndrome.¹⁸³ β -Adrenergic blockade may be most effective in the varieties of the long-QT syndrome that have involved deficient I_{Ks} and be less effective in the other varieties. Conversely, long cycle lengths may be more important as arrhythmia triggers in other varieties of long-QT syndromes, notably LQT3, and pacing may be more effective in arrhythmia suppression. Early observations indicate that LQT3 patients are more prone to sudden death at night or at rest than the other varieties of the syndrome.

The growing evidence for the roles of Ca^{2+} entry into the myocyte and Ca^{2+} loading in EAD generation has presented other possibilities for suppression of EADs and arrhythmias. Ca^{2+} -channel blockers have not had widespread use in the congenital

syndromes, but there are reports of their efficacy.²² Infusion of Mg^{2+} , a Ca^{2+} -channel blocker,¹⁸⁴ is accepted as first-line therapy for acute suppression of torsades de pointes. The commonly used cardioactive Ca^{2+} -channel blockers verapamil and diltiazem both show use-dependent block of the L-type Ca^{2+} channel,¹⁸⁵ which might limit their effectiveness at slow heart rates.

The major drawback of the powerful anti-reentrant therapeutic action of class III agents is the induction of EADs and torsades de pointes in a small percentage of patients. A desirable strategy would be to retain, even augment, the QT prolongation, but eliminate EADs and arrhythmogenesis. For this goal, interventions directed at Ca^{2+} traffic in the myocytes may be appropriate but greater understanding is required of EAD generation and ionic arrhythmia mechanisms including the putative role of M cell layers and dispersion of repolarization in reentry mechanisms that maintain torsades de pointes.^{186,187}

The trigger role of adrenergic stimulation in congenital long-QT syndromes is compatible with the hypothesis that Ca^{2+} entry, Ca^{2+} loading of the SR, and spontaneous SR Ca^{2+} release are integral components of the mechanism of EAD generation and triggering in the long-QT syndromes. However, in the acquired long-QT syndromes, β -adrenergic receptor stimulation in the form of isoproterenol has been used therapeutically to suppress torsades de pointes. One possibility for this discordance is that the effect of β -adrenergic receptor stimulation on heart rate and enhancement of I_{Ks} , as well as other repolarizing currents, may override its effect to enhance Ca^{2+} entry and Ca^{2+} loading. In the acquired long-QT syndromes, I_{Kr} may be the culprit current more commonly, allowing for action-potential shortening with adrenergic enhancement of I_{Ks} . In the case of the congenital long-QT syndromes, I_{Ks} is more commonly reduced.¹⁸³ In addition, natural adrenergic stimulation would involve stimulation of both β and α receptors. α -Adrenergic receptor stimulation may promote generation of EADs and torsades de pointes.^{188,189} β -Adrenergic blockade and left cardiac sympathectomy would be expected to reduce Ca^{2+} entry and Ca^{2+} loading and, in the case of sympathectomy, to diminish in addition α -adrenergic receptor stimulation and its possible potentiating effect on EAD generation. The clinical observation that with these therapies the QT interval is generally not normalized indicates that their role is to suppress EADs and triggering rather than affect the fundamental defect in repolarization. This concept fits with the genetic findings as well.

5. Conclusions

In conclusion, the complex ionic basis of EADs and the significance of EADs for in-vivo arrhythmogenesis initiating torsades de pointes are currently being unraveled at rapid pace. At the cellular level, the distinction between the conditional phase and the upstroke of EADs is the rationale for new experiments. Whereas the upstroke may be regarded as a passive regenerative response, the conditional phase is the target for

improved mechanistic insights and a better control. In the balance of ion fluxes creating this fundamental event during repolarization, the contribution of inactivating and reactivating inward currents is undisputed. Additionally, an important role is reserved for increased cytoplasmic and/or subsarcolemmal Ca^{2+} cycling, which may determine the 'go or no go' for EAD generation under many circumstances by setting the magnitude of inward $I_{\text{Na-Ca}}$. During systolic spontaneous Ca^{2+} release from the SR, as in some types of EADs, this magnitude is expected to be amplified. It has been demonstrated that in other types of EADs, such as those due to defective or inhibited K^+ currents, the significance of $I_{\text{Na-Ca}}$ is dependent on various momentary influences (eg, heart-rate changes and neurohumoral input), but can become arrhythmogenic even at relatively low amplitudes and in the absence of spontaneous Ca^{2+} release. It is important to recognize that the multifactorial substrate for EADs *in situ* is of a greater complexity than currently controllable by experimental design. In this regard, one should be very cautious to extrapolate the results on EADs induced under relatively steady-state conditions in the cellular electrophysiological laboratory to in-vivo arrhythmias.

From experimental and clinical investigations, it becomes increasingly clear that the various congenital and acquired long-QT syndromes may each have specific features in terms of electrophysiological substrate, inciting events for torsades de pointes, and responses to antiarrhythmic therapies. The trigger roles of dynamic rate switches and adrenergic stimulation in many of these forms are compatible with the hypothesis that Ca^{2+} entry, Ca^{2+} (over)loading of the SR, and spontaneous SR Ca^{2+} release are integral components of the mechanisms of increased dispersion of repolarization and EAD initiating ventricular tachycardia. However, this may be more pronounced in conditions in which I_{Ks} is the culprit current, than with downregulated I_{Kr} or persistent late I_{Na} when the response of a normal I_{Ks} to adrenergic stimulation and increased $[\text{Ca}^{2+}]_{\text{cyt}}$ is still sufficient to prevent excessive action-potential prolongation. The development of diagnostic electrophysiological protocols to recognize these phenotypes to tailor therapeutic strategies for the individual patient is obviously necessary.

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Summary

Under normal circumstances, the rhythmic activity of the heart is controlled by electrical impulses that originate in the sinus node and travel through the atria and the atrioventricular node to the ventricles. Via the specialized conduction system of the ventricles (bundle of His, left and right bundle branches, and Purkinje fibers), the impulses are transferred to the ventricular muscle cells. Every heart beat is the result of the synchronized activation and consequent contraction of these cells in the left and right ventricular wall, followed by the recovery from electrical activation and the relaxation from contraction, respectively.

At the level of the single myocyte, electrical stimulation during diastole initiates the action potential. Various ion fluxes crossing the membrane are responsible for the depolarization and repolarization phases of the action potential. The depolarization (phase 0) is generated mainly by the influx of Na^+ and Ca^{2+} ions, whereas the repolarization (phases 1 to 3) is determined by a large number of in- and outward currents, including inactivating Na^+ and Ca^{2+} currents, various K^+ currents, Na^+ - Ca^{2+} -exchange current, Cl^- current and Na^+ - K^+ -pump current. The resting membrane potential (phase 4) is maintained by the concerted action of the so-called inward rectifier K^+ current, the Na^+ - K^+ -pump current, and (depending on the intracellular Na^+ and Ca^{2+} concentrations) the Na^+ - Ca^{2+} -exchange current. It is likely that also other currents contribute to the resting phase.

Contraction is initiated by the influx of Ca^{2+} ions into the cell during the early phase of the action potential. This influx triggers the release of a much larger pool of Ca^{2+} stored in the sarcoplasmic reticulum. The cytoplasmic Ca^{2+} made available by this process (called ' *Ca^{2+} -induced Ca^{2+} release*') serves to activate the myofilaments causing contraction via the '*cross-bridge cycle*'. At the end of contraction, the majority of Ca^{2+} ions is taken up again by the sarcoplasmic reticulum, whereas a small fraction is extruded from the cell interior by the Na^+ - Ca^{2+} exchanger.

Under circumstances of mechanical overload, such as during hypertension, myocardial infarction, valvular disease, idiopathic hypertrophic or dilated cardiomyopathy, and complete atrioventricular block, the heart employs a number of intrinsic and extrinsic mechanisms in an attempt to maintain a normal cardiac output. One of the manifestations of chronic overload is ventricular hypertrophy, but many other complex responses occur. The contractile performance of the heart may initially show a compensatory adaptation, but, dependent on the degree and duration of overload, this is often followed by the development of some form of pump failure. In part, these contractile alterations are reducible to (mal)adaptive changes of the myocardial Ca^{2+} homeostasis.

With respect to electrophysiological alterations, consistent features observed in numerous animal models and patients with cardiac hypertrophy or failure are the prolongation of the ventricular action potential and accentuated regional differences in

action-potential durations. This may cause beat-to-beat variations of the QT interval on the electrocardiogram and latent or overt forms of acquired QT prolongation, both of which have been related to an increased risk of sudden cardiac death due to an enhanced susceptibility to ventricular tachyarrhythmias.

In the present thesis the cellular basis of acquired QT prolongation and enhanced susceptibility to the polymorphic ventricular tachyarrhythmia '*torsades de pointes*' is investigated in the dog with complete atrioventricular block of chronic duration. For this purpose, adult mongrel dogs were operated and their atrioventricular node irreversibly damaged. In the ensuing condition the conduction of electrical impulses through this node is interrupted, which causes independent beating of the atria and the ventricles. Furthermore, the heart rate drops to approximately 45 beats per minute and is fully dependent on impulse generation from the ventricles, which is intrinsically slow. As a result, the stroke volume during systole and the filling of the ventricles during diastole increase. This state of increased cardiac load causes hypertrophy of both the left and right ventricle.

Chapter 1 comprises the general introduction to the thesis and a historical overview of the congenital and acquired long-QT syndromes, and *torsades de pointes*. It is described that the ventricular arrhythmias of cardiac hypertrophy and failure appear to be based partly on pathological action-potential prolongation and increased regional dispersion of repolarization, and thus resemble the long-QT syndromes. The dog model with complete atrioventricular block is introduced and the aims of the study are put forward. The meaning of '*The Substrate*' and '*The Trigger*' is explained.

In Chapter 2 the regional heterogeneity of the action-potential configuration and duration in the normal dog heart is described. The new finding of action-potential differences between the left and right ventricular midmyocardium is further investigated in voltage-clamp studies, and appears to be attributable to differences in K^+ currents. This means that electrical remodeling of the ventricles during cardiac overload starts from uneven action-potential characteristics, which must be taken into account to explain arrhythmogenesis under these circumstances.

In Chapter 3 the cellular basis of biventricular hypertrophy and arrhythmogenesis in the dog with chronic complete atrioventricular block is examined. Ventricular enlargement is the result of cellular hypertrophy of both the left and right ventricular myocytes. In this animal model the interventricular differences of action-potential duration (known from control hearts) are exaggerated, predominantly by a pronounced prolongation of the left ventricular action potential. Midmyocardial myocytes from both ventricles exhibit an abnormal response to class III antiarrhythmic agents with much larger increases of the action-potential duration and a higher incidence of early afterdepolarizations than control cells. The data indicate that ventricular repolarization in chronic complete atrioventricular block is intrinsically weak and may predispose to class III-dependent *torsades de pointes* in vivo.

It is outlined in Chapter 4 that downregulation of certain K^+ currents contributes to the repolarization abnormalities in these dogs. In left and right midmyocar-

dial myocytes, the slowly activating component of the delayed rectifier K^+ current, which constitutes a significant fraction of the outward current underlying normal ventricular repolarization, shows markedly lower amplitudes. Furthermore, in the right ventricular cells the rapidly activating component of the delayed rectifier K^+ current is also decreased. Unlike other animal models and patients with cardiac hypertrophy or failure the transient outward K^+ current is not altered. Thus, the ionic basis of acquired QT prolongation in dogs with chronic complete atrioventricular block resembles to some extent the K^+ -current defects known for some of the human congenital long QT syndromes.

A detailed description of the cardiac contractile performance and cellular Ca^{2+} handling in chronic complete atrioventricular block is given in Chapter 5. Contractile function in vivo is enhanced, in particular at the slow idioventricular rate. Likewise, cell contraction and Ca^{2+} release from the sarcoplasmic reticulum in single left and right midmyocardial myocytes are enhanced at slow stimulation frequencies. Whereas Ca^{2+} influx through L-type Ca^{2+} channels is unchanged, Ca^{2+} influx via reverse-mode Na^+ - Ca^{2+} exchange is significantly increased and contributes to cellular Ca^{2+} loading, but also to Ca^{2+} overload. Inward-mode Na^+ - Ca^{2+} -exchange currents are increased in left (but not right) ventricular cells, which, in response to the larger Ca^{2+} transients, may cause sufficient inward current to impair a fast repolarization. Therefore, the upregulation of Na^+ - Ca^{2+} exchange may improve contractile adaptation to chronic complete atrioventricular block, but at the same time promote proarrhythmia by increasing regional dispersion of repolarization and increasing the propensity to Ca^{2+} overload and spontaneous Ca^{2+} release from the sarcoplasmic reticulum.

In Chapter 6 the cellular arrhythmogenic consequences of spontaneous Ca^{2+} release from the sarcoplasmic reticulum are investigated in normal canine ventricular myocytes. Ca^{2+} overload is induced by low concentrations of isoproterenol, which stimulates the β_1 - and β_2 -adrenergic receptors and their intracellular cascades leading to enhanced L-type Ca^{2+} currents and enhanced Ca^{2+} uptake into the sarcoplasmic reticulum. Based on contraction measurements it is concluded that spontaneous Ca^{2+} release can occur during the action potential and after completion of the repolarization, corresponding with early and delayed afterdepolarizations, respectively. These membrane responses can trigger new action potentials via various mechanisms. The results indicate that a Ca^{2+} -activated membrane current is necessary for both types of afterdepolarizations. The importance of these mechanisms lies in their capacity to generate the extrasystolic activity that may initiate torsades de pointes under circumstances of increased sympathetic activation.

Chapter 7 contains a discussion of the specific electrical alterations in the dog with chronic complete atrioventricular block and how these alterations could lead to arrhythmias. The possible pathogenetic stimuli for electrical remodeling are outlined in a schematic diagram.

Finally, in Chapter 8 the results of these studies are discussed in broader perspective, from cell to clinic. A review is given on recent advances in cardiac

arrhythmology related to afterdepolarizations. Subsequently, the emergence of new views on Ca^{2+} -dependent mechanisms of abnormal impulse generation, and their significance for the initiation and treatment of torsades de pointes are contemplated.

Samenvatting

Onder normale omstandigheden wordt de ritmische activiteit van het hart gecontroleerd door elektrische impulsen die ontstaan in de sinusknoop en die vervolgens door de boezems en de atrioventrikulaire knoop naar de kamers bewegen. Via het gespecialiseerde geleidingssysteem van de kamers (bundel van His, linker en rechter bundeltakken, en Purkinje vezels), worden deze impulsen naar de hartspiercellen van de kamers (ofwel de ventrikels) voortgeleid. Elke hartslag is het resultaat van de gesynchroneerde activatie en de daardoor geïnduceerde contractie van de hartspiercellen in de linker en rechter kamerwand, gevolgd door een herstelfase van de elektrische activatie en de relaxatie van de contractie.

In de individuele hartcel initieert elektrische stimulatie tijdens diastole de actie-potentiaal. Multiple ionenstromen die de celmembraan passeren, zijn verantwoordelijk voor de depolarisatie- en repolarisatie-fasen van de actiepotentiaal. De depolarisatie (fase 0) gebeurt voornamelijk door de influx van Na^+ - en Ca^{2+} -ionen. De repolarisatie (fasen 1 tot 3) wordt bepaald door vele in- en uitwaartse stromen, waaronder inactiverende Na^+ - en Ca^{2+} -stromen, verschillende K^+ -stromen, Cl^- -stroom, en de stromen gegenereerd door de Na^+ - Ca^{2+} -uitwisselaar en de Na^+ - K^+ -pomp. De rust-membraan-potentiaal (fase 4) wordt in stand gehouden door de zogenaamde inwaarts-rectificerende K^+ -stroom, de Na^+ - K^+ -pomp-stroom, en (afhankelijk van de intracellulaire Na^+ - en Ca^{2+} -concentraties) de Na^+ - Ca^{2+} -uitwisselaar-stroom. Het is waarschijnlijk dat ook andere stromen bijdragen aan de rustfase.

De contractie wordt geïnitieerd door de influx van Ca^{2+} -ionen in de cel tijdens de vroege fase van de actie-potentiaal. Deze influx veroorzaakt de vrijstelling van een veel grotere hoeveelheid Ca^{2+} opgeslagen in het sarcoplasmic reticulum. Het cytoplasmatische Ca^{2+} dat door dit proces (genaamd ' *Ca^{2+} -geïnduceerde- Ca^{2+} -vrijstelling*') wordt vrijgemaakt, activeert de myofilamenten, hetgeen de contractie veroorzaakt. Aan het einde van de contractie wordt de meerderheid van de Ca^{2+} -ionen weer opgenomen door het sarcoplasmatisch reticulum, terwijl een klein deel via de Na^+ - Ca^{2+} -uitwisselaar uit de cel verdwijnt.

Onder omstandigheden van mechanische overbelasting van het hart, zoals tijdens hypertensie, hartinfarct, kleplijden, idiopathische hypertrofische en gedilateerde cardiomyopathie, en compleet atrioventrikulair geleidingsblok, gebruikt het hart een aantal intrinsieke en extrinsieke mechanismen om te proberen de normale cardiale output te handhaven. Eén van de veranderingen tijdens chronische overbelasting is ventrikulaire hypertrofie, maar vele andere complexe responsen treden ook op. De contractiele eigenschappen van het hart kunnen in het begin een compensatoire adaptatie tonen, maar, afhankelijk van de mate en de duur van de overbelasting, wordt dit vaak gevolgd door de ontwikkeling van hartfalen. Deze contractiele veranderingen

zijn deels gebaseerd op (mal)adaptieve veranderingen van de myocardiale Ca^{2+} homeostase.

Ten aanzien van de elektrofysiologische veranderingen tijdens chronische overbelasting van de hartspier zijn er twee consistente bevindingen die in talloze diersoorten en patiënten met cardiale hypertrofie of falen opvallen: verlenging van de ventriculaire actiepotentiaal en toegenomen regionale verschillen in de actiepotentiaal-duur. Dit kan geaccentueerde QT-variabiliteit op het elektrocardiogram veroorzaken en latente of overte vormen van verworven QT-prolongatie. Beide zijn geassocieerd met een verhoogd risico op plotse hartdood als gevolg van een toegenomen gevoeligheid voor kamertachycardiën.

In dit proefschrift wordt de cellulaire basis van verworven QT-verlenging en verhoogde gevoeligheid voor polymorfe ventriculaire tachycardiën, de zogenaamde '*torsades de pointes*', in honden met chronisch compleet atrioventrikulair geleidingsblok onderzocht. Hiervoor werden volwassen honden geopereerd en hun atrioventriculaire geleiding onherstelbaar geblokkeerd. In deze toestand kloppen de boezems en de kamers van het hart onafhankelijk van elkaar. De hartslag daalt naar ongeveer 45 slagen per minuut en is volledig afhankelijk van impulsvorming in de kamers, welke intrinsiek traag is. Dientengevolge stijgen het slagvolume tijdens systole en de vulling van de kamers tijdens diastole. Deze staat van verhoogde cardiale belasting veroorzaakt hypertrofie van zowel de linker- als de rechterventrikel.

Hoofdstuk 1 bevat de algemene introductie van het proefschrift en een historisch overzicht van de congenitale en verworven lange-QT-syndromen, en *torsades de pointes*. Er wordt in beschreven dat de ventriculaire ritmestoornissen van cardiale hypertrofie en falen, tenminste gedeeltelijk, gebaseerd lijken te zijn op pathologische actiepotentiaal-verlenging en verhoogde regionale dispersie van de repolarisatie, en zo een gelijkenis vertonen met de lange-QT-syndromen. Het hondenmodel met compleet atrioventrikulair blok wordt geïntroduceerd en de doelstellingen van de studie geformuleerd. De betekenis van '*The Substrate*' en '*The Trigger*' wordt uitgelegd.

In Hoofdstuk 2 wordt de regionale heterogeniteit in de vorm en de duur van de actiepotentiaal in het normale hondenhart beschreven. De nieuwe bevinding van actiepotentiaal-verschillen tussen het linker- en rechterventrikel midmyocard wordt verder onderzocht in voltage-clamp-experimenten waarmee de diverse ionenstromen afzonderlijk bestudeerd kunnen worden. Verschillen in K^{+} -stromen lijken bij te dragen aan de actiepotentiaal-verschillen. Dit betekent dat de elektrische veranderingen van het kamermyoocard tijdens cardiale overbelasting reeds beginnen met regionaal ongelijke actiepotentiaal-kenmerken, hetgeen in acht moet worden genomen om de aritmogenese onder zulke omstandigheden te verklaren.

In Hoofdstuk 3 wordt de cellulaire basis van biventriculaire hypertrofie en aritmogenese in de hond met chronisch compleet atrioventrikulair geleidingsblok onderzocht. Ventriculaire vergroting is het gevolg van cellulaire hypertrofie van zowel de linker- als de rechterkamer-cellen. In dit diersoort zijn de (normaal reeds aanwezige) interventriculaire verschillen van de actiepotentiaal-duur vergroot, hoofdzakelijk door

een forse verlenging van de actiepotentiala van de linkerventrikel. Midmyocardiale cellen van beide kamers vertonen een abnormale respons op klasse-III-antiaritmica met een veel grotere verlenging van de actiepotentialen en een frequenter voorkomen van vroege nadepolarisaties dan controlecellen. De resultaten geven aan dat de ventrikulaire repolarisatie in chronisch compleet atrioventrikulair blok intrinsiek verzwakt is en kan predisponeren voor klasse-III-afhankelijke torsades de pointes in vivo.

In **Hoofdstuk 4** wordt ingegaan op een **verklaring voor de repolarisatie-afwijkingen in de hond met chronisch compleet atrioventrikulair geleidingsblok**. Het blijkt dat een afname van bepaalde K^+ -stromen hieraan bijdraagt. In linker en rechter midmyocardiale cellen, vertoont de traag-aktiverende component van de zogenaamde delayed rectifier K^+ stroom (die een significante bijdrage in de normale ventrikulaire repolarisatie heeft) opmerkelijk lagere amplitudes. Verder is in de rechterkamer cellen de snel-aktiverende component van de delayed rectifier K^+ stroom ook verlaagd. In tegenstelling tot andere diersmodellen en patiënten met cardiale hypertrofie of falen is de zogenaamde transiënte uitwaartse K^+ stroom niet verlaagd. Zo lijkt de ionische basis van verworven QT verlenging in honden met chronisch compleet atrioventrikulair blok op de bekende K^+ -stroom-afwijkingen van sommige van de humane congenitale lange-QT syndromen.

Een gedetailleerde beschrijving van de **cardiale contractiele eigenschappen en de cellulaire Ca^{2+} homeostase in honden met chronisch compleet atrioventrikulair geleidingsblok** wordt gegeven in **Hoofdstuk 5**. De contractiele functie in vivo is verhoogd, met name tijdens het trage idioventrikulaire ritme. De cellulaire contractie en Ca^{2+} -vrijstelling uit het sarcoplasmatisch reticulum in individuele linker en rechter midmyocardiale myocyten zijn eveneens verhoogd tijdens trage stimulatie-frequenties. Terwijl de Ca^{2+} -influx door L-type Ca^{2+} -kanalen onveranderd is, is de Ca^{2+} -influx via uitwaartse Na^+ - Ca^{2+} -uitwisseling significant verhoogd en draagt dit bij tot cellulaire Ca^{2+} lading, maar ook tot overlading. De inwaartse Na^+ - Ca^{2+} -uitwisselaarstroom is verhoogd in linker-, maar niet in rechterventrikelcellen en in respons op de grotere Ca^{2+} transiënten kunnen deze voldoende inwaartse stromen genereren om de repolarisatie te verzwakken. Om deze redenen kan een toegenomen Na^+ - Ca^{2+} -uitwisseling de contractiele adaptatie tijdens chronisch compleet atrioventrikulair blok verbeteren, maar op hetzelfde moment de kans op ritmestoornissen vergroten door een toename van de regionale dispersie van de repolarisatie en de neiging tot Ca^{2+} overlading en spontane vrijstelling van Ca^{2+} uit het sarcoplasmatisch reticulum.

In **Hoofdstuk 6** worden de **aritmogene consequenties van spontane Ca^{2+} vrijstelling uit het sarcoplasmatisch reticulum in normale myocyten van het hondenhart** besproken. Ca^{2+} overlading wordt geïnduceerd door lage concentraties isoproterenol. Isoproterenol stimuleert de β_1 - en β_2 -adrenerge receptoren en hun intracellulaire second messengers, hetgeen leidt tot vergrote L-type Ca^{2+} -stromen en verhoogde Ca^{2+} opname in het sarcoplasmatisch reticulum. Op basis van contractie-metingen kan geconcludeerd worden dat spontane Ca^{2+} vrijstelling tijdens en na de actiepotentiala kan optreden, en gepaard kan gaan met vroege en late nadepolarisaties. Deze

membraan-responsen kunnen via verschillende mechanismen nieuwe actiepotentialen veroorzaken. De resultaten geven aan dat een Ca^{2+} -geactiveerde membraan-stroom noodzakelijk is voor beide typen van nadepolarisaties. Het belang van deze mechanismen ligt in hun capaciteit de extrasystolische activiteit te genereren die torsades de pointes kan initiëren onder omstandigheden van verhoogde sympathische activatie.

Hoofdstuk 7 bestaat uit een discussie van de specifieke elektrische veranderingen in de hond met chronisch compleet atrioventrikulair geleidingsblok en hoe ze tot ritmestoornissen kunnen leiden. De mogelijke pathogenetische stimuli voor elektrofysiologische veranderingen worden in een schematisch diagram weergegeven.

Als laatste worden in **Hoofdstuk 8** de resultaten van deze studies in breder perspectief bediscussieerd, van cel tot kliniek. Een overzicht van de recente vooruitgangen op het gebied van de cardiale aritmologie gerelateerd aan nadepolarisaties wordt gegeven. Vervolgens worden nieuwe inzichten over Ca^{2+} -afhankelijke mechanismen van abnormale impulsvorming, en hun belang voor de initiatie en behandeling van torsades de pointes besproken.

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Curriculum vitae

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