Cardiac electrophysiological effects of some drugs applied in the treatment of arrhythmias

Ph.D. Thesis

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TABLE OF CONTENTS

Acronyms and abbreviations 6
SUMMARY 7
1. INTRODUCTION 9
1.1 Mechanisms of cardiac arrhytmias 9
1.1.1 Normal automaticity 9
1.1.2 Abnormal automaticity 10
1.1.3 Early afterdepolarizations and triggered activity 10
1.1.4 Delayed afterdepolarization-induced triggered activity 10
1.1.5 Reentrant arrhythmias 11
1.2 Early repolarization syndrome 12
1.2.1 Diagnosis and risk stratification of early repolarization syndrome 12
1.2.2 Epidemiology of early repolarization syndrome 15
1.2.3 Cellular electrophysiology of early repolarization pattern 16
1.2.4 Correlations between J wave syndromes and hypothermia 17
1.2.5 Genetic background of early repolarization syndrome 19
1.2.6. Parasympathetic influences on early repolarization syndrome 19
1.3 Different electrophysiological effects of mexiletine stereoisomers 21
1.4 Aims of the study 22
2. METHODS 23
2.1. Arterially perfused wedge of canine left ventricle 23
2.2. Measurements of AP parameters and J wave area calculations 24
2.3. Conventional microelectrode technique 25
2.4. Statistical analysis 26
3. RESULTS 27
3.1 Early repolarization syndrome and hypothermia. 27
3.1.1 Pharmacologic modeling of genetic mutations associated with early repolarization syndrome 27
3.1.2 Mechanism underlying arrhythmogenic influence of parasympathetic tone in ERS 27
3.1.3 Effects of hypothermia in left ventricular wedge preparation 31

3.1.4 Effects of hypothermia in ER-induced left ventricular wedge preparation 32
3.1.5 Effects of quinidine to suppress and prevent hypothermia-induced arrhythmogenesis 34
3.1.6 Effects of phosphodiesterase III inhibitors to suppress and prevent hypothermia-induced arrhythmogenesis. 36
3.2 Effects of mexiletine enantiomers on transmembrane action potentials 40
4. DISCUSSION 46
4.1 Investigation of J-wave related arrhythmias in normo- and hypothermia and therapeutical possibilities 46
4.2 Investigation of mexiletine enantiomers 49
5. CONCLUSION 53
Study Limitations 54
Acknowledgements 54
References 55

LIST OF FULL PAPERS RELATED TO THE SUBJECTS OF DISSERTATION

1. **Gurabi Z**, Koncz I, Patocskai B, Nesterenko VV, Antzelevitch C.
Cellular mechanism underlying hypothermia-induced ventricular tachycardia/ventricular fibrillation in the setting of early repolarization and the protective effect of quinidine, cilostazol, and milrinone.
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2. Koncz I, **Gurabi Z**, Patocskai B, Panama BK, Szél T, Hu D, Barajas-Martínez H, Antzelevitch C.
Mechanisms underlying the development of the electrocardiographic and arrhythmic manifestations of early repolarization syndrome.
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Acronyms and abbreviations

ACh	acetylcholine
APA	action potential amplitude
APD50	action potential duration at 50% of repolarization
APD90	action potential duration at 90% of repolarization
BCL	basic cycle length
BrS	Brugada syndrome
CHF	congestive heart failure
CT	conduction time
ECG	electrocardiogram
EDR	epicardial dispersion of repolarization
ER	early repolarization
ERP	early repolarization pattern
ERS	early repolarization syndrome
ENDO	endocardial
EPI	epicardial
IVF	idiopathic ventricular fibrillation
LQTS	long QT syndrome
LV	left ventricle
MAP	monophasic action potential
MDP	maximum diastolic potential
NI	notch index
NM	notch magnitude
PDE	phosphodiesterase
PH	phase
RV	right ventricle
SCD	sudden cardiac death
S.E.M.	standard error of the mean
SR	sarcoplasmic reticulum
TdP	torsades de pointes
TDR	transmural dispersion of repolarization
VF	ventricular fibrillation
VMAX	maximal rate of depolarization
VT	ventricular tachycardia

SUMMARY

An early repolarization (ER) pattern in the ECG is characterized by a J point elevation in 2 contiguous inferior and/or lateral ECG leads other than V1-V3 with or without ST-segment elevation, and it manifests sometimes as a notch or slur on the terminal part of QRS complex. Early repolarization pattern, long described to be benign, was recently proposed to have a malignant component on the basis of the association of ER with vulnerability to ventricular fibrillation (VF)/ventricular tachycardia (VT). Recent studies demonstrated that therapeutic hypothermia can unmask undiagnosed ER and may provoke VT/VF. There is a need for drugs able to prevent the hypothermia-induced VT/VF developing in the setting of ER. There is necessity to examine the cellular mechanisms underlying VT/VF associated with hypothermia in an experimental model of ER syndrome (ERS) and to examine the effectiveness of quinidine, cilostazol and milrinone to prevent hypothermia-induced arrhythmias.

Mexiletine, the widely used antiarrhytmic drug, is applied as a racemic preparation to treat acquired long QT syndrome-related torsades de pointes (TdP) tachyarrhythmias and other extracardiac disorders, i.e. Timothy-syndrome, neuropathies and myotonic disorders. The detailed cardiac electrophysiological effects of the R-(-) and S-(+) mexiletine isomers on rabbit cardiac ventricular preparations have not been investigated yet**.**

We aimed to test the hypothesis that ER, by causing outward shift in the balance of currents active during the early phases of the epicardial action potential (AP), exacerbates the response to hypothermia, thus leading to prominent J waves, phase 2 reentry and VT/VF. In addition, we aimed to examine the hypothesis that pharmacologic agents capable of producing an inward shift in the balance of current in the early phases of the epicardial AP, either by inhibiting transient outward current (Ito) (quinidine) or augmenting ICa (cilostazol and milrinone), can protect against hypothermia-induced VT/VF in the setting of ER. Transmembrane action potentials (AP) were simultaneously recorded from 2 epicardial and 1 endocardial site of coronary-perfused canine left-ventricular wedge preparations, together with a pseudo-ECG.

Our next aim was to study the effect of the R-(-) and S-(+) mexiletine on rabbit cardiac action potential parameters (action potential duration (APD), conduction time (CT) and maximal rate of depolarization (Vmax)) by using the conventional microelectrode technique.

Our results demonstrate that hypothermia leads to VT/VF in the setting of ER by exaggerating repolarization abnormalities, leading to development of phase-2-reentry. Quinidine, cilostazol

and milrinone suppress the hypothermia-induced VT/VF by reversing the repolarization abnormalities.

We found that R-(-) mexiletine displays slower offset kinetics than S-(+) mexiletine, and R-(-) mexiletine displays a tendency to more potent inhibitory effect than S-(+) mexiletine on Vmax, especially at early premature action potentials, i.e.: this enantiomer might exert stronger antiarrhythmic effect especially in terminating early ventricular extrasystoles.

1. INTRODUCTION

1.1 Mechanisms of cardiac arrhytmias

A cardiac arrhythmia can be defined as a deviation from the physiological heart rhythm. An arrhythmia can be regular, as in the case of monomorphic tachycardia or flutter; or it can be irregular as in the case of fibrillation or polymorphic tachycardia. Some of them may be benign, e.g premature ventricular contractions (PVC); while others are often seen to be malignant, e.g ventricular fibrillation - a commonly seen causative agent of sudden cardiac death. The most prevalent sustained arrhythmia in the clinic is atrial fibrillation. The responsible mechanisms of arrhythmias are commonly divided into two major categories: abnormal impulse formation and reentry. Reentry develops when a propagating impulse fails to expire after physiologic activation and reactivates cells after the refractory period (Antzelevitch et al. 2008).

1.1.1 Normal automaticity

Automacity is defined as the ability of cardiac cells to create action potentials. Spontaneous acitivity is generated by diastolic depolarization that is necessary to reach the threshold of membran potential. Under physiologic conditions the sinoatrial node has the highest intrinsic rate. Subsidiary pacemakers initiate excitation only if the sinoatrial node fails to generate impulses or when impulses fail to propagate (Hope et al. 1976; Vassalle 1977). The main ionic mechanism that are responsible for sinoatrial, atrioventricular and Purkinje automaticity are hyperpolarization-activated inward current (I_f) (DiFrancesco 1985; DiFrancesco 1995) and/or decay of outward potassium current (IK) (Vassalle1977). The rate at which impulses are generated is determined by the following factors (Hoffman and Cranefield 1960): maximum diastolic potential, threshold potential and slope of phase 4 depolarization. Parasympathetic activation decreases the spontaneous rate via the activation of potassium current (IK-ACh), IK-ACh reduces inward Ca^{2+} current (I_{Ca}) and the pacemaker current (I_f). Furthermore, acetylcholine hyperpolarizes the cell, leading to an increase in maximum diastolic potential and slows the phase 4 depolarization. Sympathetic activation/beta adrenergic agonists increase the spontaneous rate via the augmentation of inward calcium current (I_{Ca}) and the pacemaker current (I_f).

1.1.2 Abnormal automaticity

Abnormal, depolarization-induced automaticity is usually observed when the resting membran potential is reduced, e.g ischemia, infarction or other depolarizing influences. The membrane potential at which abnormal automaticity develops ranges between -70 and -30 mV (Hauswirth et al. 1969). In abnormal activity, heart rate is higher compared to normal activity, and it can be provoked by beta adrenergic activation or by reduction of external potassium (Imanishi and Surawicz 1976; Katzung and Morgenstern 1977). The more depolarized membrane potential (that is associtated with diseases) is the result of the following effects: increased extracellular K^+ concentration - that reduces the potential of IK_1 outward current; reduced number of IK_1 channels; reduced ability of IK_1 channels to conduct potassium ions and the electronic influence of the neighboring depolarized zone (Antzelevitch et al. 2008).

1.1.3 Early afterdepolarizations and triggered activity

Early afterdepolarizations (EAD) are commonly associated with prolongation of the repolarization as a result of a reduced net outward current due to increased inward and/or decreased outward currents. EAD occurs usually when inward currents become more accentuated compared to baseline conditions (Trautwein 1970). The following factors can generate early afterdepolarization: reduction of repolarizing potassium current (e.g blockade of IK_r by class IA and III antiarrhytmic agents); increase in the availability of calcium current (e.g by catecholamines); enhanced sodium-calcium current exchange current due to augmented intracellular calcium activity or upregulated exchanger; augmented late sodium current. EAD-induced extrasystoles are often seen to be responsible in the generation of torsade de pointes arrhythmias in congenital or acquired long QT syndromes (Roden et al. 1996; Antzelevitch et al. 2000). EAD-like deflections were detected before TdP arrhythmias developed in ventricular MAP recodings as well as in experimental models of LQTS (mainly in LQT2, 3 and 6) (Carlsson et al. 1992; Ben-David and Zipes 1988; Jackman et al. 1988; Shimizu et al. 1991; Asano et al. 1997). The prolongation of action potentials are presented in cases of myocardial hypertrophy and heart failure.

1.1.4 Delayed afterdepolarization-induced triggered activity

Delayed afterdepolarization appears during phase 4 and it can manifest as an accompanying aftercontraction. This type of afterdepolarization is thought to be the result of transient inward current (I_{ti}) that made either by nonselective cationic current (I_{ns}) (Kass et al. 1978; Cannell

and Lederer 1986), or activated Na/Ca exchanger (Kass et al 1978; Fedida et al. 1987; Laflamme and Becker 1996; Zygmunt et al. 1998), or calcium activated Cl^- current. These factors lead to release of Ca^{2+} from SR (Laflamme and Becker 1996; Zygmunt et al. 1998). Delayed afterdepolarization (DAD) and DAD-triggered activity are presented under conditions with increased intracellular calcium concentration, such as toxic levels of cardiac glycosides or catecholamines (Rozanski and Lipsius 1985, Wit and Cranefield 1977, Priori and Corr 1990; Marchi et al. 1991), hypertrophy of myocardium or heart failure. Potassium concentration is also appeared to influence DADs, because K^+ concentration under 4mM promotes DAD, as well as lisophosphatidylcholine and increased extracellular ATP level are capable to provoke DADs (Pogwizd et al. 1986; Song and Belardinelli 1994) while high K^+ concentration abolishes DADs (Wit and Rosen 1992; Coetzee and Opie 1987). Prolongation of the action potential duration produced by e.g quinidine can also facilitate DADs via the augmented calcium influx. Low concentration of caffeine can generate DADs via the exaggregated calcium release from SR. Agents that inhibit calcium current, e.g verapamil or flunarizine are capable to suppress DADs (Gorgels et al. 1990; Tytgat et al. 1988; Vos et al. 1990; Park JK et al. 1992; Chattipakorn and Ideker 2003; Kass et al. 1978).

1.1.5 Reentrant arrhythmias

In circus movement reentry mechanism, an electrical impulse spreads devious around an anatomical or functional obstacle and that precipitates the reexcitation of the heart. For the development of this type of reentry, two factors are necessary: a precipitating extrasystole that can be either automatic, triggered or reentrant and a substrate that is usually the result of electrical and structural heterogeneities. Phase 2 reentry about which further details will be shown in the present dissertation is another type of a reentrant mechanism that seems to be of focal origin. Phase 2 reentry arrhythmia appears when the dome of the action potential propagates from regions at which it was maintained to regions at which it was lost. This leads to a re-excitation and can generate closely coupled extra-systoles, ventricular tachycardia or fibrillation associated with Brugada syndrome and early repolarization syndrome (Antzelevitch and Burashnikov 2011).

1.2 Early repolarization syndrome

1.2.1 Diagnosis and risk stratification of early repolarization syndrome

The appearance of J waves was associated earlier with hypothermia (Clements et al. 1972; Thompson et al. 1977; Eagle et al. 1994), hypercalcaemia (Kraus 1920; Sridharan 1984) and inherited and acquired cardiac arrhythmia syndromes (Antzelevitch and Yan 2010). The elevated J wave/J point in ECG was demonstrated firstly by Tomaszewski in 1938 in an accidentally frozen human (Tomaszewski 1938). J wave is also known as Osborn wave since Osborn investigated this phenomenon in hypothermic dogs (Osborn 1953).

In some animal species, distinct J waves can be seen under baseline conditions, e.g. in dogs or in baboons, while in humans the distinct J wave can be rarely observed under physiologic circumstances, however, an elevated J point is commonly encountered. An early repolarization (ER) pattern in the ECG is characterized by a J point elevation in 2 contiguous inferior and/or lateral ECG leads other than V1-V3 with or without ST-segment elevation, and it manifests sometimes as a notch or slur on the terminal part of QRS complex. Early repolarization pattern, long described to be benign, was recently proposed to have a malignant component on the basis of the association of ER with vulnerability to ventricular fibrillation/ventricular tachycardia in humans and in experimental models consisting of canine ventricular wedge preparations, thus identifying the early repolarization syndrome (ERS) (Huikuri 2016; Wasserburger and Alt 1961; Mehta and Jain 1995). J waves are commonly seen to appear in ’notched’ or ’slurred’ phenotype. Notched J wave is a notching at the second half of downslope of R-wave. Slurred J wave appears as a slowing of the wave form at the terminal part of QRS complex and it is usually in a fusion with ST-segment, however, a distinct J wave has been described to have three parts (Jo: onset of the J wave, Jp: peak of the J wave and Jt, referred as a termination of J wave). The exact diagnosis of early repolarization pattern requires a J wave peak (Jp) $\geq$0.1 mV, a QRS-complex that lasts $\geq$0.12 mV and the onset of the J wave (or the peak of the J wave in slurred ones) must be above zero-line (Macfarlane et al. 2015).

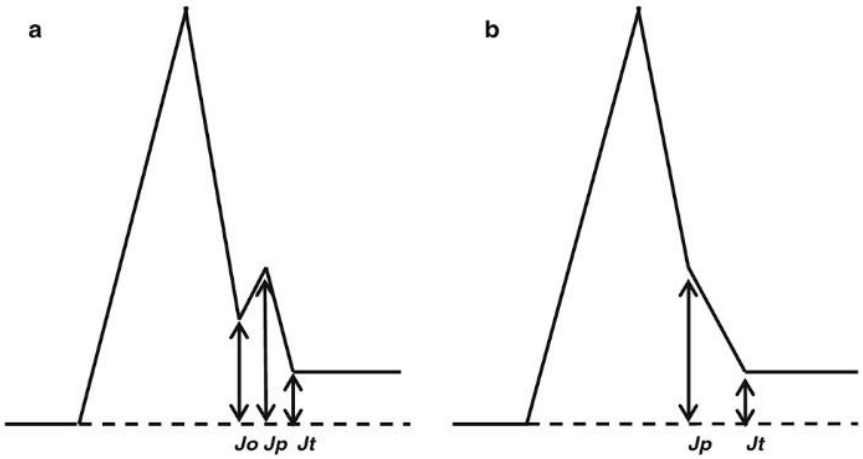

Figure 1. *Illustration of notched (A) and slurred type (B) J wave. Jo: onset of J wave, Jp: peak of the J wave, Jt: termination (end) of J wave. (From: Huikuri HV. Prevalence and risk stratification of patients with electrocardiographic pattern of early repolarization. In: J wave Syndromes. Edited by Charles Antzelevitch and Gan-Xin Yan. Springer International Publishing Switzerland. 2016. page 195.)*

In the case of downsloping or horizontal ST segment, the amplitude of the ST-segment 100 ms after the termination of the J wave is smaller or the same as the amplitude of the J wave (Figure 1.). This type of ST segment slope is considered to have an increased risk of developing VT/VF. Ascending ST segment has been seen when 100 ms after the termination of the J wave, the amplitude of ST-segment is higher than the amplitude of Jt (Figure 1.). Ascending ST segment elevation is described to have a lower risk of developing ventricular tachyarrhythmia (Macfarlane et al. 2015). The vital role of J waves in the development of VT/VF has been proven by several studies (Shu et al. 2005; Bjerregaard 1994; Yan and Antzelevitch 1996; Geller et al. 2001; Daimon et al. 2000; Kalla et al. 2000; Komiya et al. 2006; Shinohara et al. 2006; Riera et al. 2004). Haissaguerre et al. described in their study that 31% of their patients with idiopathic ventricular fibrillations showed early repolarization pattern (ERP) where ERP was defined as elevation of the QRS-ST junction of > 0.1 mV and manifested as QRS slurring or notching (Haissaguerre et al. 2008). Nam et al. published similar findings: in their study, 60% of the patients with idiopathic ventricular fibrillation (IVF) exhibited ERP on the ECG (Nam et al. 2008). In J wave syndromes, a male predominance was observed (Antzelevitch and Yan 2010; Shimizu et al. 2007); and it was proven by Barajas-Martinez et al. that testosterone acts as an agonist on I_{to}, which is a key point in the development of J wave syndromes (Barajas-Martinez et al. 2013).

J WAVE SYNDROMES*				
Type of J Wave Syndromes	**Early Repolarization Syndrome Type 1**	**Early Repolarization Syndrome Type 2**	**Early Repolarization Syndrome Type 3**	**Brugada syndrome**
Anatomic Location	Antero-lateral left ventricle	Inferior left ventricle	Left and right ventricles	Right ventricle
Leads Displaying J wave / J-point elevation	I., V_4-V_6	II., III., aVF	Global	V1-V3
Risk for developing VT/VF	Low; Commonly seen in healthy athletes	Higher than in Type 1	ERP with the highest risk for developing VT/VF	High risk for developing VT/VF
Sex dominance	Male	Male	Male	Male

Table 1. Classification of J wave syndromes. *Modified from: Antzelevitch C and Yan GX. J wave syndromes. Heart Rhythm. 2010 Apr;7(4):549-58.*

J wave syndromes are divided into four subtypes. Table 1 summarizes the main properties of each subtypes. Type 1 involves an early repolarization pattern localized to the lateral leads. In Type 2, the early repolarization pattern is presented in the inferior and inferolateral leads, and in Type 3 it is presented globally in the inferior, lateral and anterior or right precordial leads. Individuals with Type 1 rarely develop VT/VF. Type 2 is associated with a much higher risk for the development of ventricular tachycardia and fibrillation (VT/VF) (Tikkanen et al. 2009; Junttila et al. 2012) compared to Type 1. Individuals, displaying a Type 3 ER, have the highest risk for developing malignant arrhytmias. In Brugada syndrome, the J point elevation and/or ST-segment elevation are seen only in right precordial leads. Brugada syndrome is associated with a relatively high risk for developing ventricular fibrillation or ventricular tachycardia. Early repolarization syndrome (ERS) and Brugada syndrome (BrS) represent together the J wave syndromes. Both of them are associated with the development of VT/VF leading to sudden cardiac death in young adults (Antzelevitch and Yan 2010; Haissaguerre et al. 2008; Nam et al. 2008, Brugada P and Brugada J 1993) without structural heart diseases. ERS and BrS show regional differences but display clinical similarities and share similar pathophysiology (Yan and Antzelevitch 1999; Yan and Antzelevitch 1996; Antzelevitch 2013; Nam 2012; McIntyre et al. 2012), however, differences were also described e.g. in the response to sodium channel blockers: reduction of J waves in ERS but accentuation of J wave

in the case of BrS (Kawata et al. 2012) after the addition of sodium channel blockers. Main similarities of the two syndromes are the follows: male predominance (Benito et al. 2008; Kamakura et al. 2013; Haissaguerre et al. 2008); both of the syndromes can be totally asymptomatic until syncope or sudden cardiac arrest develop often secondary to VF (Antzelevitch and Yan 2010); incidence of VT/VF is the highest in the third decade of life when testosterone level is high. During bradycardia, sleep (Kawata et al. 2012) and following long pauses (Kalla et al. 2000; Aizawa et al. 2012), J wave and ST segment elevation are more prominent and VT/VF are seen more often.

Early repolarization pattern (ERP) and early repolarization syndrome (ERS) must be distinguished. ERP is the ECG finding discussed above in the abscence of symptomatic arrhythmias. If ERP is accompanied with a history of resuscitated idiopathic VF and/or polymorphic ventricular tachycardia (VT), early repolarization syndrome (ERS) is diagnosed. (Priori et al. 2013). It was calculated that the risk of developing VT/VF is 11:100000 in patients with J wave on the ECG compared to 3.4:100000 in patients without J wave (Rosso et al. 2008; Rosso et al. 2011). In the presence of early repolarization pattern, highly predictive can be for VT/VF: (1.) previous syncope or cardiac events due to VT/VF, (2.) pause dependent augmentation of J waves, especially when it appears together with T wave inversion (Aizawa et al. 2012; Qi et al. 2004), (3.) prominent J waves in global leads, as well as ST segment elevation in the right precordial leads (Priori et al. 2012, Antzelevitch et al. 2005), (4.) association of BrS or ER pattern with abbreviated QT intervals (Burashnikov et al. 2010; Watanabe et al. 2010), (5.) short-coupled extrasystoles (Komiya et al. 2006; Gang et al. 2004), (6.) nocturnal agonal respiration, (7.) T wave variability (Yoshioka et al. 2013), (8.) short ventricular refractory period (VRP <200 ms) or (9.) fragmented QRS (Morita et al. 2008;Priori et al. 2012), (10.) prolonged QRS duration (Junttila et al. 2008).

1.2.2 Epidemiology of early repolarization syndrome

Tikkanen et al. investigated the prevalence of early repolarization pattern on 12-lead electrocardiography in a community-based general population. Early repolarization pattern of ≥0.1 mV was presented at 5.8% of all subjects. ERP appeared 3.5% in inferior leads and 2.4% in lateral leads. Elevation at both, inferior and lateral leads were seen in 0.1% of all subjects. J-point elevation of ≥0.2 mV occured in 0.5% (Tikkanen et al. 2009). Klatsky et al. found in their study that early repolarization pattern showed a male predominance, especially in athletes and it was seen more frequently in black people than in whites or in Asians. Interestingly, those with early repolarization had slightly lower body mass index, systolic and

diastolic blood pressures, total blood cholesterol and glucose levels and do recreational exercises more often than those without ERP (Klatsky et al. 2003).

1.2.3 Cellular electrophysiology of early repolarization pattern

The cellular background of the development of J wave in the ECG has been subject to discussion for a long time. It was described that under physiological conditions a more prominent I_{to}-mediated action potential notch in ventricular epicardium but not in the endocardium leads to a transmural voltage gradient that appears as a J wave. Further tests have strengthened this hypothesis (i.e. the size of the J wave is I_{to} mediated), e.g premature stimulation or tachycardia generated a parallel decrease in the amplitude of the I_{to}-mediated epicardial action potential notch due to the slow reactivation kinetics of I_{to} after a premature beat, thus it decreased the transmural voltage gradient and J wave shrinked (Koncz et al. 2013). Quinidine or 4-aminopyridin (4-AP), the direct inhibitor of I_{to} current also decreased the magnitude of J wave via the reduction of epicardial action potential notch. Additionally, the end of phase 1 of epicardial action potential is coincident with the peak of the J-point elevation (Yan and Antzelevitch 1996). Agents that reduce I_{Ca} e.g. verapamil or intensify I_{to}, such as NS5806 (direct activator of I_{to}) or hypothermia have an opposite effect (Gurabi et al. 2014; Antzelevitch and Yan 2000; Yan et al. 2003; Calloe et al. 2009; Fish and Antzelevitch 2004; Koncz et al. 2014): they increase the size of J wave/ elevate the J point. In early repolarization pattern, an accentuated AP notch is a commonly seen phenomenon in the LV epicardium due to increased net repolarizing currents: because of *1. decreased inward currents* or *2. increased outward currents*. When ERP is presented, a further increase in net repolarizing currents and a further accentuation of epicardial AP notch (e.g due to cholinergic agonists or hypothermia) can lead to a loss of AP dome at some epicardial sites but not others thus creating a significant transmural voltage gradient that manifests as accentuated J wave with or without ST-segment elevation on the ECG. When action potential (AP) dome is lost at some sites, AP dome can propagate from regions at which it was maintained to sites at which it was lost leading to a local re-excitation via phase 2 reentry mechanism (Yan and Antzelevitch 1999; Antzelevitch and Yan 2000; Yan et al. 2003; Koncz et al. 2014). The voltage gradient between *1. intact action potential* and *2. action potential with loss of the dome morphology* creates the transmural and epicardial dispersion of repolarization. When phase 2 reentry catches a vulnerable window generated by epicardial or transmural dispersion of repolarizations through ventricular wall, it can provoke premature ventricular complexes and VT/VF (Koncz et al. 2014). It was demonstrated that augmentation of the I_{to} current due

to genetic abnormalities, bradycardia, increased vagal tone or pharmacologic agents produces J wave elevation (Calloe et al. 2009; Koncz et al. 2014; Gurabi et al. 2014; Patocskai et al. 2016). Many studies have described yet, that the inhibition of I_{to} current by tachycardia or by pharmacological agents such as 4-AP – discussed above, quinidine, milrinone, cilostazol or with isoproterenol is capable to lower or abolish action potential notch and J wave (Koncz et al. 2014; Gurabi et al. 2014; Patocskai et al. 2016). The features of J wave is predominantly I_{to}-mediated, however, other additional ionic currents such as reduction/ inhibition of I_{CaL}, I_{Na} and the enhancement of $I_{K\text{-}ATP}$, $I_{K\text{-}ACh}$ and I_{Kr} are also seemed to be important mediators of transmural repolarization heterogenity in ER (Antzelevitch and Yan 2015).

1.2.4 Correlations between J wave syndromes and hypothermia

The electrocardiographic J wave (also known as Osborn wave) has long been recognized as pathognomonic of hypothermia (Antzelevitch and Yan 2010; Osborn 1953) and it is considered to be able to provoke ventricular tachycardia or fibrillation. The extent to which hypothermia contributes to arrhyhmogenesis and the mechanisms involved are not well defined. Several studies were performed for the better understanding of the ionic background of hypothermia in heart. Antzelevitch and Yan investigated the effect of hypothermia on ventricular wedge preparations (Antzelevitch and Yan 1995). They found that the decrease in the temperature of the perfusate of wedge modells caused an increase in the amplitude and width of the action potential notch in epicardium but not in endocardium. This report showed that differences in the response to hypothermia in the early phases of repolarization of epicardial and endocardial sites of the ventricles (i.e. augmenting the action potential notch in epicardium but not endocardium) resulted in a transmural voltage gradient that increased the amplitude of the J wave. The ionic basis for the augmentation of the epicardial action potential notch was indicated to be due to differences in the Q10 (temperature coeffitient) for the kinetics of I_{Ca} and I_{to}. At low temperature, this difference would be expected to cause a greater cooling-induced slowing of I_{Ca} activation than of I_{to} activation leading to a very significant outward shift in the balance of current during the early phases of the action potential (Fish and Antzelevitch 2004; Dumaine et al. 1999; Gurabi et al. 2014).

Recent studies have investigated the importance of hypothermia in the treatment of ongoing ischaemia in acute cases such as stroke, acute myocardial infarction and cardiac arrest or after reperfusion (Lampe and Becker 2011); current guidelines recommend mild therapeutic hypothermia to prevent neurological damage e.g following a cardiac arrest. (American Heart Association 2006; Castrén et al. 2009). Therapeutic hypothermia seems to be protective

against ischaemia-reperfusion injury via the reduction of cellular metabolism (Erecinska 2003), attenuation of abnormal free radical production (Shao et al. 2010), optimization of cellular pH balance (Polderman 2009), reduction of cell death and inflammatory signaling (Yang et al. 2009). The American Heart Association recommended therapeutic hypothermia between 32℃ and 34℃ for 12 to 24 hours to treat comatose survivors of cardiac arrest (2005 American Heart Association guidelines for cardiopulmonary resuscitation and emergency cardiovascular care. Part 7.5: Postresuscitation support.). Trials have shown that therapeutic hypothermia can improve significantly the long-time survival after a cardiac arrest (Hypothermia after Cardiac Arrest Study Group 2002; Bernard et al. 2002). De Georgia et al. examined endovascular cooling in patients with ischaemic stroke. By patients who underwent hypothermia, the mean diffusion-weighted imaging (DWI) lesion growth was smaller compared with control group (De Georgia et al. 2004). A recent study has also observed the effect of therapeutic cooling in patients with anterior ST-elevation myocardial infarction (STEMI) without cardiac arrest. Intravascular temperature was decreased to 33.6 °C in 22 subjects. Median infarct size/left ventricular mass ratio was 16.7% in the hypothermia group versus 23.8% in the control group, furthermore, median left ventricular ejection fraction (LVEF) was 42% in the hypothermia group and 40% in the control group (Noc et al. 2017). In recent reports an interaction between ER and the development of VT/VF in the setting of hypothermia were reported. Bastiaenen et al. reported earlier that a 38-year old man with inferior early repolarization pattern presented series of ventricular fibrillations (VFs) during therapeutic hypothermia applied after the patient suffered an out of hospital cardiac arrest (Bastiaenen et al. 2010). An other case report was described by Federman et al. showed a 34-year-old man having inferolateral ER who suffered hypothalamic injury leading to body temperature fluctuation. The patient had ventricular fibrillation at 32 ºC. Later the patient was treated with therapeutic hypothermia for neurologic protection but below 35 ºC several ventricular fibrillation occured (Federman et al. 2013). Our study was designed to explore the mechanisms underlying ventricular fibrillations in patients with ER at hypothermia, and to establish possible therapeutic agents to be applied in this severe condition. To create ER phenotype, we used the NS5806 (3-10 μM) which is a transient outward potassium current (I_{to}) agonist (Calloe et al. 2010), verapamil (1 μM) which is a blocker of L-type calcium channels and acetylcholine (3μM). By adding this combination of drugs, we were able to mimic the increased transient outward potassium current mutation (Perrin et al. 2014; Calloe et al. 2009), decreased inward calcium current current (Burashnikov et al. 2010) and increased vagal tone (Wilhelm et al. 2010; Marcus et al. 2002; Mizumaki et al. 2012; Mizumaki et al.

2004; Shinohara et al. 2017). To explore possible therapeutic agents in hypothermia induced ventricular fibrillation or tachycardia, we tested three drugs: quinidine, and two phosphodiesterase (PDE) type III inhibitors (cilostazol and milrinone). The effectiveness of quinidine in the treatment of ER has recently been shown in a multicentre study (Haïssaguerre et al. 2009). Cilostazol has recently been reported to be able to suspend ER associated VF in long term treatment (Iguchi et al. 2013). Milrinone, the other PDE type III inhibitor has not been reported yet to be beneficial in early repolarization syndrome.

1.2.5 Genetic background of early repolarization syndrome

The inheritable nature of early repolarization pattern was proved by studies earlier (Noseworthy et al. 2011; Reinhard et al. 2011; Nunn et al. 2011). ER is assiociated with the mutations of several genes. Loss of mutations of genes (CACNA1C, CACNB2, and CACNA2D1) that are responsible for α1, β2 and α2δ subunits of the cardiac L-type calcium channel are associated with early repolarization syndrome (Burashnikov et al. 2010). Loss of function mutation of I_{Na} current, e.g. SCN5A (Watanabe et al. 2011) and SCN10A (Hu et al. 2014) gene mutations are also associated with ER. Gain of function mutations in *KCNJ8* or in *ABCC9*, the genes responsible for the pore forming subunit of the ATP-sensitive potassium channel (K_{ATP}), are associated with ERS; they are often seen genetic bases of early repolarization syndrome. $I_{K\text{-}ATP}$ channel activations in canine ventricular wedge preparations led to ER pattern (Koncz et al. 2014; Barajas-Martinez et al. 2012; Medeiros-Domingo et al. 2010; Haissaguerre M, Chatel S et al. 2009). Perrin et al. described that the gain of function mutation of I_{to} encoding KCND2 gene can be also responsible for J wave syndrome associated sudden cardiac death (Perrin et al. 2014).

1.2.6. Parasympathetic influences on early repolarization syndrome

Cholinergic nerve fibers innervate the ventricles and this innervation is functionally significant (Bailey et al. 1979). Recently Ulphani et al. nicely made visible the parasympathetic innervation of porcine atria and ventricles. They used histochemical method to demonstrate the presence of acetylcholinesterase; in this manner cholinergic nerves were stained white. Their findings proved that the epicardial and endocardial surfaces of ventricles are richly innervated by parasympathetic nerves, and nerve density was greater on the ventricular endocardium, but nerve thickness was greater on the epicardium (Ulphani et al. 2010). In another study, it was also described that cardiomyocytes are capable of producing acetylcholine. By using immunogold electron microscopy, rat cardiomyocytes seemed to be

able to express choline acetyltransferase (ChAT) in the cytoplasm. Furthermore, vesicular acetylcholine transporter with the vesicular structure were also identified, suggesting that cardiomyocytes have components for ACh synthesis. In addition, exogenous ACh or pilocarpine aggravated the transcriptional activity of the ChAT gene through a muscarinic receptor and ChAT protein expression; and elevated the intracellular ACh level (Kakinuma et al. 2009). Moreover, Rocha-Resende et al. investigated the cardioprotective effect of cardiomyocytes-produced-ACh against hypertrophic adrenergic effects. They found that ACh is capable of moderating/ceasing the deleterious effects of hyperadrenergic stimulation, i.e hypertrophic effect as well as molecular changes and calcium transient alterations induced by adrenergic overstimulation. On the other hand, they also described that adrenergic stimulation can enhance the expression of cholinergic components (Rocha-Resende et al. 2012). Many studies observed the effects of parasympathetic influences on early repolarization pattern. Wilhelm et al. examined the impact of parasympathetic activation on ER in professional soccer players and they found that parasympathetic influence was more dominant in subjects by whom early repolarization pattern appeared on ECG. Subjects with ERP on ECG presented a significant lower heart rate (Wilhelm et al. 2010). Patients with spinal cord injury were also examined in point of the appearance of early repolarization pattern on their ECG. T5 or above located injuries were compared to T6 or below located injures and to control patients. In cases where the central sympathetic command of the heart was disrupted due to the injury (high-level injury group - T5 or above), the loss of the sympathetic tone caused a significantly higher ST segment elevation compared to ECG recordings of low-injury patients and control patients (Marcus et al. 2002). Mizumaki et al. investigated relations between J wave amplitude elevation and changes in RR interval or autonomic nervous activities in patients with idiopathic ventricular fibrillation (IVF). They found that in patients with IVF compared to control subjects, J wave was elevated in a larger extent when parasympathetic activation increased e.g. during bradycardia or at night during sleep; and it was associated with increased arrhythmic activity i.e spontaneous ventricular fibrillation (Mizumaki et al. 2012). Similar results were described in Brugada patients where spontaneous augmentation of ST elevation in daily life occurred along with an increase in vagal activity. Under similar vagal tone, ST segment elevation was augmented more in patients with ventricular fibrillation than in patients without ventricular fibrillation (Mizumaki et al. 2004). Shinohara et al. examined patients with implantable cardioverter defibrillators to prove whether there is any connection between ventricular fibrillation and exaggerated parasympathetic tone. In their measurements, among others, the increased baroreflex sensitivity represented the enhanced parasympathetic

reactivity: baroreflex sensitivity was significantly higher in the recurrent-VF group than in the nonrecurrent-VF group (Shinohara et al. 2017). Kodama et al. reported a case history when an 51-year-oldman experienced an aborted sudden cardiac death. No structural heart diseases were found by routine noninvasive cardiac examinations and coronary angiography did not reveal any vasospastic findings. During left coronary infusion of ACh, the surface ECG demonstrated distinct J wave elevation in inferior leads without chest pain. Short coupled repetitive premature ventricular beats and further growth in J wave elevation appeared due to the increase in the dose of acetylcholine from 10 to 100 μg. These actions presumably based on the direct actions of acetylcholine (Kodama et al. 2017)**.**

1.3 Different electrophysiological effects of mexiletine stereoisomers

Mexiletine, which is an orally active congener of lidocaine with longer half-life, was described earlier to have class I.B antiarrhythmic effect (Vaughan Williams 1998). Mexiletine is capable to influence the function of the heart by blocking sodium channels with relatively fast onset and offset kinetics of Vmax (Campbell 1983; Varró et al. 1985), by reducing use-dependently the magnitude of fast sodium current (Hering et al. 1983), by slowing premature conduction (Hohnloser et al. 1982), by suppressing abnormal automacity in Purkinje fibers (Sarkozy and Dorian 2006) and by shortening the durations of action potentials (Arita et al. 1979; Yamaguchi et al. 1979).

The main cardiac indication of the non-selective voltage-gated sodium channel blocker mexiletine is the treatment of ventricular arrhythmias (Singh et al. 1990; Mason et al. 1993) and it is peculiarly beneficial in the third type of long QT syndrome (Funasako et al. 2016). A recent article dealt with the potentiability of mexiletine to prevent torsades de pointes tachycardia in acquired long QT syndrome (Badri et al. 2015). Moreover, the mexiletine is applied efficiently in extracardiac disorders, too. It was proven to have local anaesthetic actions and it is widely administered in the therapy of myotonic disorders (Logigian et al. 2010), in Timothy syndrome (Gao Y et al. 2013), in neuropathies (O'Connor et al. 2009) and in chronic pain (Park and Moon 2010).

Nowadays racemic mexiletine preparations, that contain both isomers (R- and S- mexiletine), are used therapeutically. De Luca et al. performed a study that demonstrated the stereoselective effects of mexiletine enantiomers on sodium currents and excitability of skeletal muscle fibers (De Luca et al. 1995). However, the electrophysiologic effects of the R- or S-isomer alone on cardiac cells were not investigated yet.

1.4 Aims of the study

The aim of the present study was to examine the cellular mechanisms underlying VT/VF associated with hypothermia in a canine experimental model of ER syndrome and to investigate the effectiveness of quinidine, cilostazol, and milrinone to prevent hypothermia-induced arrhythmias. Another goal of the present investigation was to study the effects of R- and S-mexiletine stereoisomers on maximum rate of depolarization (Vmax), conduction time and repolarization in isolated rabbit cardiac preparations.

2. METHODS

All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, Revised 1996).

2.1. Arterially perfused wedge of canine left ventricle

Our wedge preparation protocols were approved by the Institutional Animal Care and Use Committee. We used mongrel dogs weighing 20–35 kg of either sex. The chest was opened via a left thoracotomy and the heart was excised. Transmural wedge preparations with dimensions of up to 32x20x15 mm were dissected from the LV wall. The preparations were cannulated via a distal diagonal branch of the left anterior descending coronary artery, or a left marginal branch of the circumflex artery, or a branch of the posterior descending artery and perfused with cardioplegic solution (Tyrode's containing 12 mmol/L KCl). Non-perfused regions of the tissue were removed using a razor blade. The preparations were then placed in a tissue bath and perfused with oxygenated Tyrode's solution (mM): NaCl 129, KCl 4, NaH_2PO_4 0.9, $NaHCO_3$ 20, $CaCl_2$ 1.8, $MgSO_4$ 0.5, glucose 5.5, pH 7.4. The perfusate was delivered using a peristaltic pump (Masterflex peristaltic pump, Cole Parmer Instrument Co, Niles, Illinois) at a constant flow rate at 12-14 mL/min warmed to 37 ± 0.5°C. The preparations were equilibrated in the tissue bath until electrically stable, usually 1 hour. Pacing stimuli were delivered to the endocardial surface basic cycle length of 1000 ms using bipolar silver electrodes insulated except at the tips.

The temperature of the perfusate was controlled by a heating bath associated with a glass condenser, a tube internally coiled within a wide cylindrical housing. The Tyrode's solution was warmed while passing through the heated coils to deliver the perfusate at 37°C. In hypothermia protocols to stimulate hypothermia, the solution was redirected to two coiled-perfusion lines in series immersed in beakers filled with water, before reaching the tissues. We lowered the temperature of the perfusate to 32°C. A transmural ECG was recorded using two electrodes consisting of AgCl half cells placed in the tissue bath, 1.0 to 1.5 cm from the Epi and Endo surfaces of the preparation, along the same axis as the transmembrane recordings (Epi electrode is connected to the positive input of the ECG amplifier). Transmembrane APs were simultaneously recorded from two Epi sites (Epi 1 and Epi 2; Epi1-Epi2 distance was approx. 10-20 mm) and one Endo site with the use of floating

microelectrodes (DC resistanc e=10 to 20 MΩ) filled with 2.7 mol/L KCl, each connected to a high-input impedance amplifier. Impalements were obtained from the Epi and Endo surfaces of the preparation at positions approximating the transmural axis of the ECG recording. Spike 2 for Windows (Cambridge Electronic Design, Cambridge, UK) was used to record and analyze the ECG and the AP. NS5806, cilostazol and milrinone were dissolved in dimethyl sulphoxide (DMSO); acetylcholine, verapamil HCl, quinidine were dissolved in distilled water (10 mM stock). DMSO controls were performed to ensure the absence of an effect of the solvent.

2.2. Measurements of AP parameters and J wave area calculations

The epicardial AP notch magnitude (phase 1 magnitude/ phase 0 amplitude × 100), phase 0 to phase 2 interval (time between the first 2 peaks of the derivative of the AP), as well as the notch index (notch magnitude × [Ph 0–Ph 2 interval]), which approximates the area of the notch, were measured in AP recordings as previously described (Figure 2.). The area of the J wave was calculated as follows: the start of J wave was defined using derivative of the ECG signal. In case of clear separation, it was set at the time when this derivative is zero which corresponds to the notch between R wave and J wave. When this separation was not clearly visible, this time was set at the moment when the negative derivative attains its maximal value (i.e. minimal rate of decline) after the maximal downslope of the R wave. The J wave area was expressed as millivolt × millisecond (Figure 2.).

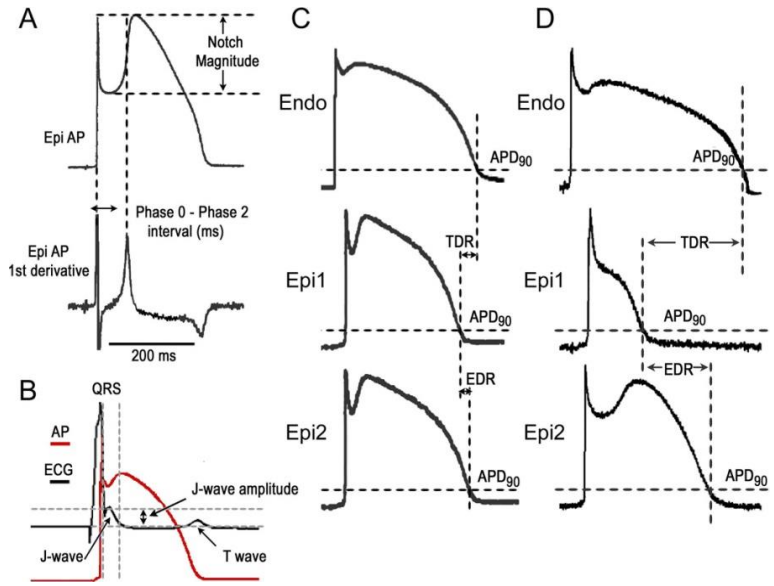

Figure 2. A and B: *Method of quantitation of action potential (AP) and J wave parameters.***C:***Measurement of dispersion of AP repolarization.***D:***Measurement of dispersion*

of repolarization when the AP dome is lost at 1 epicardial (Epi) site but not the other. APD90 indicates action potential duration at 90% repolarization; EDR, epicardial dispersion of repolarization; Endo, endocardial; and TDR, transmural dispersion of repolarization (from Gurabi et al. 2014).

2.3. Conventional microelectrode technique

Conventional microelectrode measurements were approved by the Review Board of the Committee on Animal Research of the University of Szeged (54/1999 OEj). Young male New Zealand rabbits (1000–2000 g) were euthanized by a blow on the neck and the hearts were removed. Free right ventricular wall and the right papillary muscles were prepared and placed into the tissue bath (50 mL) and allowed to equilibrate for at least 1 h while superfused (flow rate 4–5 mL/min) with Locke's solution containing (in mmol/L): NaCl 120, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 22, and glucose 11. The pH of this solution was 7.40 –7.45 when gassed with 95% O_2 and 5% CO_2 37 °C. During the equilibration period, the ventricular muscle tissues were stimulated at a basic cycle length of 1000 ms. Electrical pulses of 2 ms in duration and twice diastolic threshold in intensity (S_1) were delivered to the preparations through bipolar platinum electrodes. Transmembrane potentials were recorded with the use of glass capillary microelectrodes filled with 3 mol/L KCl (tip resistance: 5–15 MΩ). The microelectrodes were coupled through an Ag–AgCl junction to the input of a high impedance, capacitance-neutralizing amplifier (MSB-MET Ltd). Intracellular recordings were displayed on a storage oscilloscope (Hitachi V-555) and led to a computer system (APES) designed for online determination of the following parameters: resting membrane potential, action potential amplitude, action potential duration at 10%, 25%, 50%, and 90% repolarization, and the maximum rate of rise of the action potentialupstroke (Vmax). The following types of stimulation were applied in the course of the experiments: stimulation with a constant cycle length of 1000 ms (ventricular muscles); stimulation with different constant cycle lengths ranging from 300 to 5000 ms. To establish the recovery of Vmax, extra test action potentials were elicited by using single test pulses (S_2) in a preparation driven at a basic cycle length of 1000 ms (S_1). The S_1–S_2 interval was gradually increased from the end of refractory period. The action potential characteristics of the potentials evoked by each S_2 were determined. The diastolic interval preceding the test action potential was measured from the point corresponding to 90% repolarization of the preceding basic action potential to delivery of S_2. The effective refractory period (ERP) of the preparation, defined as the shortest S_1–S_2 interval that evoked a propagated action potential using a stimulus amplitude equal to twice the

diastolic threshold intensity, was also determined. Once control measurements had been obtained, the preparation was superfused either for 45 min with 20 μmol/L R(−)-mexiletine or S(+)-mexiletine. The therapeutically and experimentally relevant concentration of mexiletine amounts to about 20 μmol/L (Paalman et al. 1977). Same concentration was applied at both stereoisomers. Following these periods of exposure, all action potential parameters were again obtained.

2.4. Statistical analysis

Results were expressed as means ± S.E.M. throughout our study. In Tables 2., 8. and 9. statistical comparisons were made using Student's *t*-test. Statistical comparisons were made using Student's *t*-test for effect of hypothermia on action potential duration at 90% of repolarization, maximum epicardial dispersion of repolarization, transmural dispersion of repolarization, notch index, notch magnitude, notch duration, and J wave area and effect of hypothermia on these parameters in the setting of ER (Tables 3. and 4.). One-way repeated measures ANOVA was used for effects of quinidine, cilostazol, and milrinone (Tables 5., 6. and 7.), followed by pairwise comparisons corrected by Bonferroni method.

3. RESULTS

3.1 Early repolarization syndrome and hypothermia.

3.1.1 Pharmacologic modeling of genetic mutations associated with early repolarization syndrome

Pharmacologic modeling of the genetic defects known to underlie ERS was evaluated in initial series of experiments. We sought to examine the effects of I_{Ca} inhibition and I_{to} augmentation on AP and ECG characteristics of coronary-perfused wedge preparations isolated from the inferior and lateral regions of canine LV. Loss of function of I_{Ca} mutations were pharmacologically modeled using the calcium channel blocker verapamil (3 μM) and the I_{to} agonist NS5806 (7 μM) to accentuate the epicardial AP notch and to induce J-point elevation, thus generating a prominent ER pattern. This combination of drugs produced a net outward shift in the balance of current in the early phases of the epicardial AP, causing marked accentuation of epicardial AP notch, thus giving rise to a prominent ER, heterogeneous loss of the AP dome, and the development of phase 2 reentry-induced VT/VF. Similar heterogeneous loss of the AP dome leading to development of phase 2 reentry-induced VT/VF was recorded in 5 of 10 wedge preparations isolated from the LV wall of the canine heart. (Figure 3. and Table 2.)

3.1.2 Mechanism underlying arrhythmogenic influence of parasympathetic tone in ERS

Vagal influences are known to accentuate the ECG and arrhythmic manifestations in patients with ERS. In the next series of experiments, we examined the basis for these effects by exposing wedge preparations sensitized with a combination of 7 μM NS5806 and 3 μM verapamil to ACh. Figure 4 shows an example in which the combination of NS5806 and verapamil caused marked accentuation of the epicardial AP notch, thus inducing J-point and ST segment elevation, but no arrhythmia developed. Addition of ACh (3 μM), to mimic increased vagal tone, accentuated epicardial AP notch magnitude, increased notch index, facilitated loss of the epicardial AP dome (Table 2.), enhanced ST segment elevation and precipitated repeated episodes of phase 2 reentry and polymorphic VT/VF. Similar exacerbation of arrhythmic activity by ACh was observed in 5 out of 11 wedge preparations isolated from LV wall of the canine heart that failed to exhibit arrhythmic activity when exposed to NS5806 (7 μM) and verapamil (3 μM) alone.

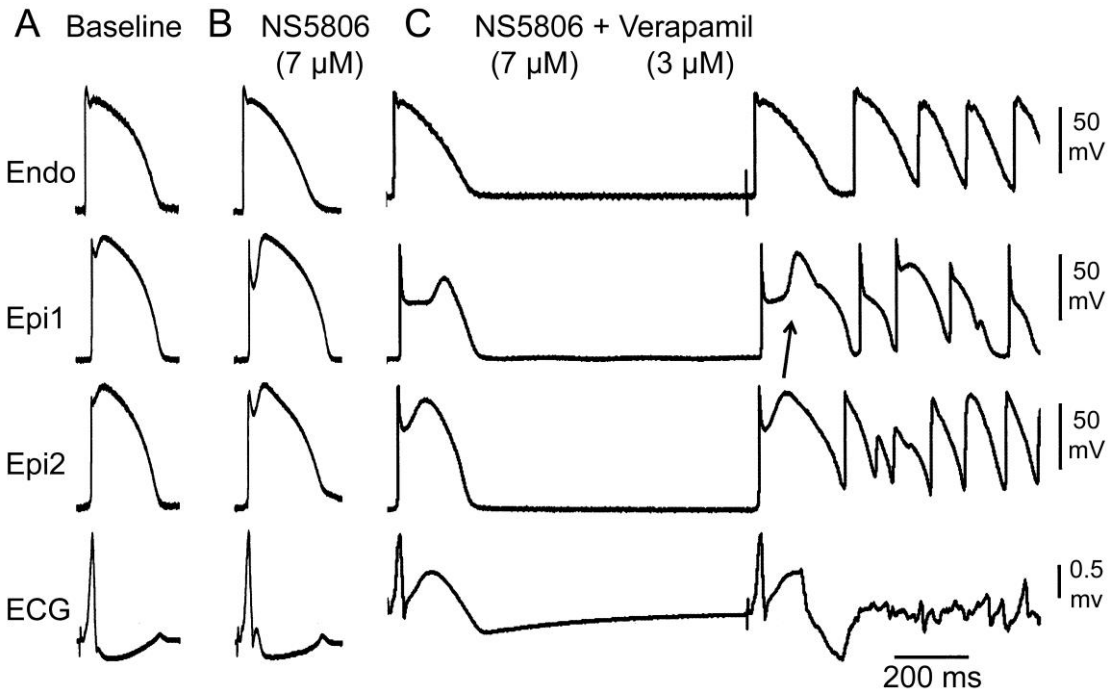

Figure 3. *The application of the calcium channel inhibitor verapamil together with I_{to} agonist NS5806 leads to development of phase 2 reentry and polymorphic ventricular tachycardia (VT) in a left ventricular wedge preparation. Action potentials from one endocardial site (Endo) and two epicardial sites (Epi1 and 2) were recorded stimoultaneously together with pseudo-ECG.* ***A:*** *Control;* ***B:*** *NS5806 (7 μM) accentuated the Epi action potential (AP) notch causing the appearance of a distinct J wave.* ***C:*** *Addition of verapamil (3 μM) to wedge perfusate mimicked the loss of function calcium channel mutations and caused a more accentuated epicardial AP notch and J wave, and a more elevated ST-segment. Phase 2 reentry and polymorphic VT/ventricular fibrillation developed 15 min after addition of verapamil. BCL = 1000 ms.*[Koncz et al. 2014]

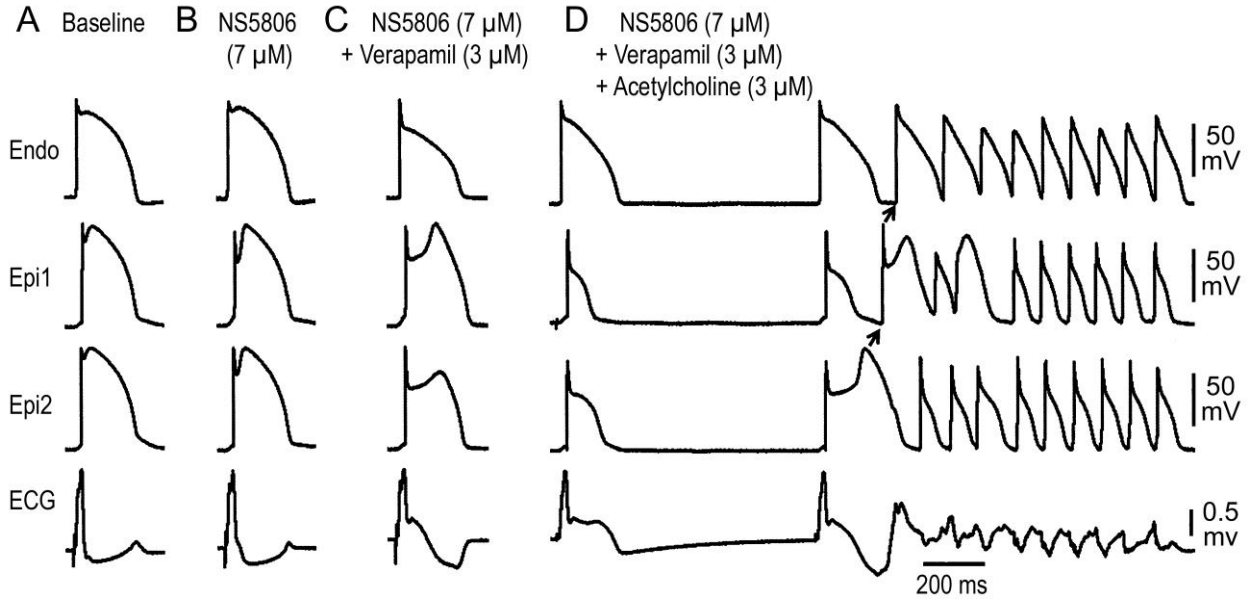

Figure 4. *Addition of acetylcholine (ACh) leads to development of phase 2 reentry and polymorphic ventricular tachycardia (VT). Action potentials from one endocardial site (Endo) and two epicardial sites (Epi1 and 2) were recorded stimoultaneously together with pseudo-ECG.* ***A:*** *Control;* ***B*** *and* ***C:*** *The combination of verapamil (3 μM) and transient outward current agonist (NS5806, 7 μM)* ***D:*** *The addition of ACh, used to mimic increased vagal tone (ACh, 3 μM), led to the development of phase 2 reentry and polymorphic VT/ventricular fibrillation. Basic Cycle Length = 1000 ms.*[Koncz et al. 2014]

	Endo APD90 (ms)	Epi1 APD90 (ms)	Epi2 APD90 (ms)	Notch Magnitude (% ofPh0)*	Notch Index†	TDR (ms)	EDR (ms)
Control	223.2 ± 2.1	199.7 ± 5.9	201.8 ± 3.9	14.0 ± 3.1	255.2 ±54.3	17.0 ± 3.5	14.7 ± 2.8
NS 5806 (7 μM)	226.2 ± 5.1	203.7 ± 6.5	207.2 ± 2.6	31.7 ± 4.0	780.8 ±116.0	17.7 ± 5.3	13.3 ± 2.8
Verapamil (3 μM)	227.0 ± 7.9	225.9 ± 8.3[a]	222.5 ± 4.6[a]	38.9 ± 5.0[b]	2551.9 ±182.8[b]	21.3 ± 4.4	14.6 ± 6.4
Acetylcholine (3 μM)‡	217.3 ± 3.1	129.9 ± 17.2[c]	246.3 ± 26.6	42.2 ± 5.1	4654.5 ± 894.0	80.8 ± 15.5[c]	111.2 ± 24.1[c]

Table 2. *Effects of provocative agents on transmural and epicardial (Epi) dispersions of repolarization and on action potential (AP) parameters in perfused left ventricular wedge*

*preparations. APD90 = action potential durations at 90% repolarization. Results are mean ± S.E.M. a=p<0.05, b=p0.01 vs control, c=p<0.05 vs NS5806 + verapamil combination. Basic cycle length = 1000 ms; n = 5. *Epicardial AP notch data (notch magnitude and notch index) measured before loss of the AP dome at epicardial sites. †Notch index = Notch magnitude × (Ph 0 to Ph 2 interval) which approximates the area of the notch. ‡ Epicardial dispersion of repolarization (EDR) and transmural dispersion of repolarization (TDR) reported here were measured in cases at which the addition of Acetylcholine caused loss of the AP dome at EPI 1 site but no EPI 2, with the development of phase 2 reentry.* [Modified from: Koncz et al. 2014]

3.1.3 Effects of hypothermia in left ventricular wedge preparation

In an initial series of 7 experiments, we examined the effects of hypothermia in coronary-perfused canine LV wedge preparations. Lowering the temperature to 32-34˚C caused prolongation of AP durations (APDs) and accentuation of the AP notch in the epicardium but not in endocardium, thus augmenting the amplitude of the J wave. Under baseline condition, lowering the temperature to 32ºC failed to cause loss of the Epi AP dome or to induce arrhythmic activities (Figure 5. and Table 3.).

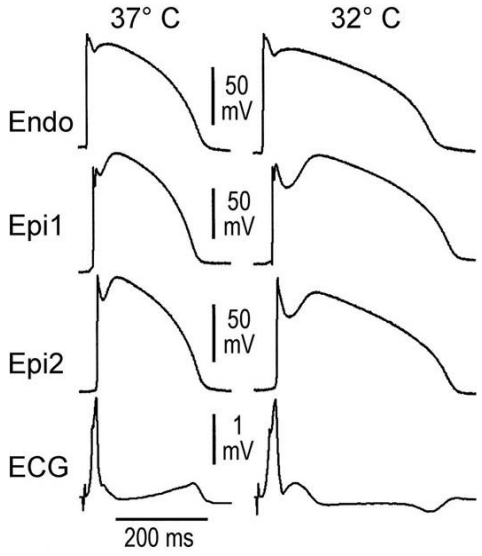

Figure 5. *Effects of lowering the temperature (from 37°C to 32°C) of the coronary perfusate in isolated canine left ventricular preparations under baseline conditions. Action potential traces simultaneously recorded from 2 epicardial (Epi) and 1 endocardial (Endo) sites together with a pseudo-ECG at 37°C and at 32°C.* [Gurabi et al. 2014]

	Endo APD90 (ms)	Epi1 APD90 (ms)	Epi2 APD90 (ms)	EDR (ms)	TDR (ms)	Notch Index*	Notch magnitude (% of Ph0)	Notch-duration (ms)	J wave area (mV × ms)
Control (37°C)	224.1 ± 2.7	200.3 ± 5.9	194.2 ± 5.5	15.1 ± 5.9	13.0 ± 4.4	180.0 ± 19.1	12.2 ± 3.0	20.1 ± 1.5	1.6 ± 0.6
Control (32°C)	320.7 ± 10.7[b]	306.3 ± 18.1[b]	295.7 ± 13.9[b]	20.0 ± 9.9	26.3 ± 8.5	1918.3 ± 393.3[b]	39.0 ± 3.7[b]	47.1 ± 5.9[b]	10.8 ± 3.8[b]

Table 3. *Effects of hypothermia on action potential duration at 90% of repolarization (APD90), maximum epicardial (Epi) dispersion of repolarization (EDR), transmural dispersion of repolarization (TDR), notch index, notch magnitude, notch duration and J wave area. *Notch index= Notch magnitude × (Ph 0 to Ph 2 interval) which approximates the area of the notch. Results are mean ± S.E.M. a=p<0.05, b=p<0.01 32°Celsius vs. 37°Celsius. n=7* [Gurabi et al. 2014].

3.1.4 Effects of hypothermia in ER-induced left ventricular wedge preparation

Because vagal influences are known to accentuate the electrocardiographic and arrhythmic manifestations of ER (Gross 2010; Mizumaki et al. 2012; Gourraud et al. 2013) in the next series of experiments, we induced an early repolarization phenotype using a combination of the I_{to} activator NS5806 (3-10 μM), the Ca^{2+} channel blocker verapamil (1 μM) and acetylcholine (ACh) (3 μM) added to the coronary perfusate. The combination accentuated the AP notch in epicardium but not endocardium, thus leading to augmentation of the electrocardiographic J wave but no arrhythmia (Figure 6). Hypothermia caused a further increase of J wave area, NM and notch index, leading to all-or-none repolarization at the end of phase 1 of the Epi AP. Loss of the Epi AP dome at some sites but not others resulted in a prominent increase in epicardial dispersion of repolarization (EDR) and transmural dispersion of repolarization (TDR). The voltage gradient between the abbreviated Epi AP and the relatively normal Endo AP produced a prominent ST segment elevation. A prominent APD gradient developed between sites displaying a normal AP dome and adjacent sites where the dome was lost, thus creating a vulnerable window within epicardium as well as between epicardium and endocardium across the ventricular wall. Propagation of the dome from regions at which it was maintained to regions, at which it was lost, caused local re-excitation via a phase 2 reentry mechanism, leading to the development of closely coupled extrasystoles and polymorphic VT/VF (Figure 6).

Similar results were obtained in 12 experiments. Composite of the action potential and electrocardiographic parameters are presented in Figure 6 and Table 4.

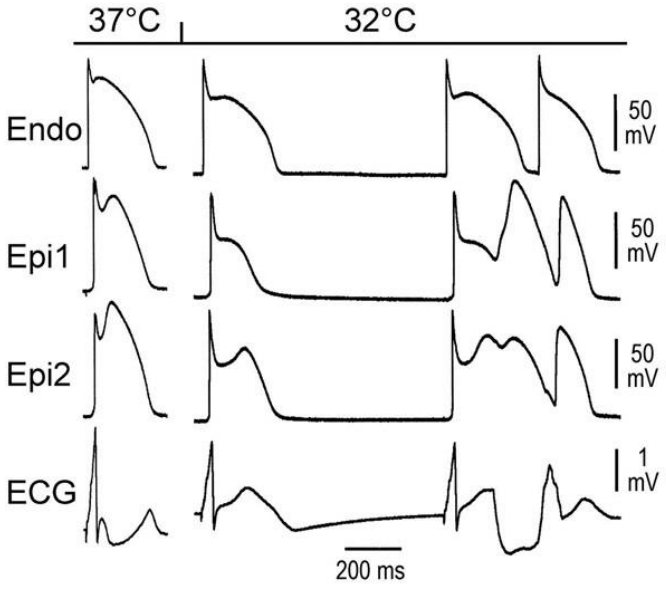

Figure 6. *Arrhythmogenic effect of hypothermia in the setting of early repolarization. Action potential traces simultaneously recorded from 2 epicardial (Epi) and 1 endocardial (Endo) sites together with a pseudo-ECG. At first, we recorded action potentials 30 minutes after the addition of NS5806 (10 µmol/L), verapamil (1 µmol/L), and acetylcholine (ACh; 3 µmol/L) at 37°Celsius. Cooling the temperature to 32°Celsius leads to the development of phase 2 reentry and VF/VT.* [Gurabi et al. 2014]

	Endo APD90 (ms)	Epi1 APD90 (ms)	Epi2 APD90 (ms)	EDR (ms)	TDR (ms)	Notch Index*	Notch magnitude (% of Ph0)	Notch-duration (ms)	J wave area (mV × ms)
Provocative agents† (37°C)	234.5 ± 4.8	209.1 ± 4.7	212.2 ± 3.9	5.6 ± 1.7	11.5 ± 2.7	1163.9 ± 140.8	30.9 ± 2.2	36.5 ± 2.5	9.6 ± 1.0
Provocative agents† (32°C)	353.0 ± 18.2[b]	154.5 ± 14.2[a]	298.9 ± 18.4[b]	140.0 ± 17.4[b]	181.9 ± 22.2[b]	4435.2 ± 286.4[b]	42.0 ± 2.2[a]	106.5 ± 5.2[b]	62.3 ± 11.3[b]

Table 4. *Effects of hypothermia in the setting of ER on action potential duration at 90% of repolarization (APD90), maximum epicardial (Epi) dispersion of repolarization (EDR), transmural dispersion of repolarization (TDR), notch index, notch magnitude, notch duration and J wave area.*Notch index= Notch magnitude × (Ph 0 to Ph 2 interval) which approximates the area of the notch. †Provocative agents= NS5806 3-10 µM + Verapamil 1*

μM + Acetylcholine 3 μM. Results are mean ± S.E.M. a=p<0.05, b=p<0.01 32°Celsius vs. 37°Celsius. n=12 [Gurabi et al. 2014].

3.1.5 Effects of quinidine to suppress and prevent hypothermia-induced arrhythmogenesis

Quinidine has been shown to restore transmural electrical homogeneity and abort arrhythmic activity in the J wave syndromes (Haïssaguerre et al. 2009). In another series of 5 experiments we tested the hypothesis that quinidine could prevent the hypothermia-induced VT/VF developing in the setting of ER owing to its effect to reduce the I_{to}. Ventricular tachycardia/ventricular fibrillation was first induced by exposure of the LV wedge to hypothermia + NS 5806 (3-10 μM) + verapamil (1μM) + ACh (3 μM). Temperature was then restored to 37℃, at which point the arrhythmia subsided. Quinidine (5 μM) was then added to the coronary perfusate and hypothermia was re-induced. Quinidine (5 μM) diminished the AP notch and J wave at 37℃ and prevented loss of the Epi AP dome and development of the repolarization abnormalities, thus preventing the development of phase 2 reentry and VT/VF when temperature was reduced to 32℃ (Figure 7). A similar effect of quinidine to prevent VT/VF was observed in 5 out of 5 preparations exposed to hypothermia (Figure 7, Table 5). In 2 experiments, quinidine (5-10 μM) was added during the VT/VF episode at 32℃. In both cases the drug suppressed all arrhythmic activity and restored electrical homogeneity throughout the preparation.

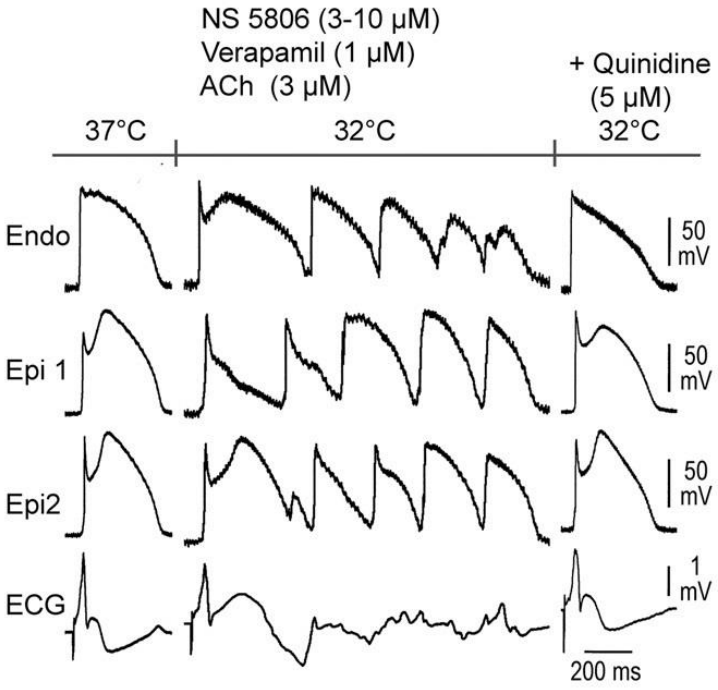

Figure 7. *Arrhythmogenic effect of hypothermia in the setting of early repolarization and the preventive effect of quinidine. Action potential traces simultaneously recorded from 2 epicardial (Epi) and 1 endocardial (Endo) sites together with a pseudo-ECG. NS5806 (3–10 μmol/L)+verapamil (1 μmol/L)+acetylcholine (ACh; 3 μmol/L) were given to the perfusate then temperature was lowered to 32°C and VT/VF developed. After the restorement of the temperature to physiologic, the arrhythmia disappeared (not shown). Quinidine was capable to prevent arrhythmic events, ECG and action potential abnormalities after the reinduction of hypothermia*. [Gurabi et al. 2014]

	Endo APD90 (ms)	Epi1 APD90 (ms)	Epi2 APD90 (ms)	EDR (ms)	TDR (ms)	Notch Index*	Notch magnitude (% of Ph0)	Notch-duration (ms)	J wave area (mV × ms)
Control (37°C)	225.9 ±2.7	195.3 ±5.2	197.2 ±7.0	10.9 ±2.6	15.3 ±5.7	250.5 ±92.2	12.3 ±4.3	20.5 ±1.9	1.6 ±0.9
Provocative agents† (37°C)	230.1 ±6.8	212.7 ±7.2	212.3 ±4.5	4.6 ±2.1	11.4 ±3.1	1107.5 ±201.2	28.3 ±3.4[c]	38.4 ±3.0	11.1 ±2.4
Provocative agents† (32°C)	343.9 ±30.4[b]	125.4 ±7.6[b]	278.2 ±24.3[a]	152.4 ±21.0[b]	201.5 ±31.5[b]	3823.4 ±217.7[b]	41.7 ±3.9	94.6 ±9.9[b]	75.4 ±22.2[a]
+Quinidine (5 μM) (32°C)	369.9 ±15.1	352.0 ±16.3[f]	349.6 ±16.4[e]	4.1 ±1.5[f]	21.5 ±11.0[f]	1783.8 ±361.3[f]	25.3 ±3.9[e]	69.1 ±4.8[e]	14.9 ±4.4[e]

Table 5. *Effects of hypothermia in the setting of ER on action potential duration at 90% of repolarization (APD90), maximum epicardial (Epi) dispersion of repolarization (EDR), transmural dispersion of repolarization (TDR), notch index, notch magnitude, notch duration and J wave area and the effect of quinidine on these parameters. *Notch index= Notch magnitude × (Ph 0 to Ph 2 interval) which approximates the area of the notch. †Provocative agents= NS5806 3-10 μM + Verapamil 1 μM + Acetylcholine 3 μM. Results are mean ± S.E.M. a=p<0.05,b=p<0.01 32°Celsius vs. 37°Celsius; c=p<0.05 NS5806 3-10 μM + Verapamil 1 μM + Acetylcholine 3 μM (37°Celsius) vs. Control (37°Celsius); e=p<0.05,f=p<0.01 Quinidine 5 μM (32°Celsius) vs. NS5806 3-10 μM + Verapamil 1 μM + Acetylcholine 3 μM (32°Celsius*). [Gurabi et al. 2014]

3.1.6 Effects of phosphodiesterase III inhibitors to suppress and prevent hypothermia-induced arrhythmogenesis.

In a final series of experiments, we examined the effectiveness of the phosphodiesterase III inhibitors cilostazol and milrinone to prevent hypothermia-induced VT/VF in the setting of ER. These agents are known to augment I_{Ca} via their action to increase cAMP. Here again, we first demonstrated the ability of the combination of provocative agents and hypothermia (32℃) to accentuate ER, thus creating a large epicardial and transmural dispersion of repolarization giving rise to phase 2 reentry and VT/VF. We then restored temperature to 37℃, which reversed the repolarization abnormality and suppressed VT/VF. The addition of cilostazol (10 μM) or milrinone (5 μM) reduced the Epi AP notch at 37℃ and prevented the repolarization abnormality as well as the development of phase 2 reentry and VT/VF when temperature was once again reduced to 32℃ (Figures 8 and 9). Both agents prevented the hypothermia-induced increase in TDR, EDR; Epi AP notch magnitude, notch index, and J wave area on pseudo ECG. Cilostazol was successful in preventing the VT/VF in 5 out of 7 preparations. Similarly, milrinone (5 μM) prevented the hypothermia-induced VT/VF in 5 out of 7 preparations (Figures 8 and 9, Table 6 and 7).

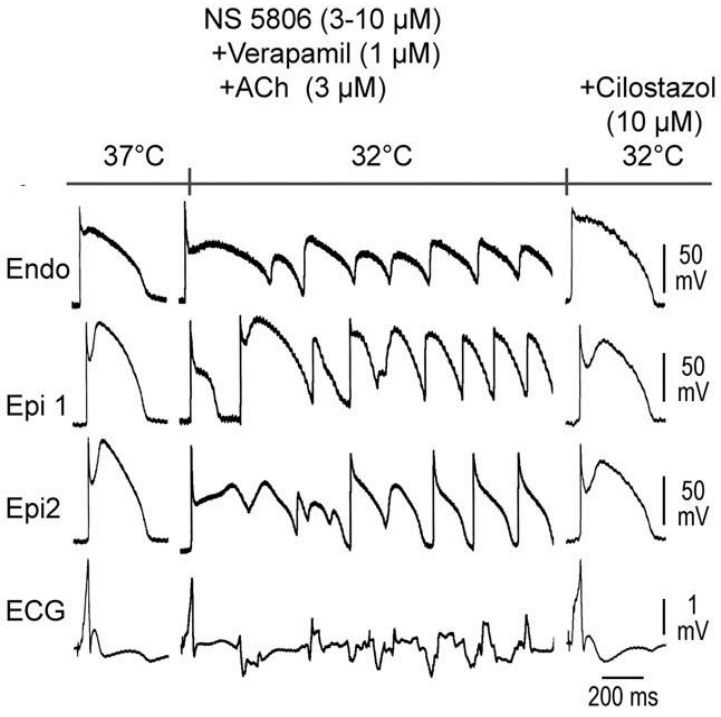

Figure 8. *Arrhythmogenic effect of hypothermia in the setting of early repolarization and the preventive effect of cilostazol. Action potential traces simultaneously recorded from 2 epicardial (Epi) and 1 endocardial (Endo) sites together with a pseudo-ECG. NS5806 (3–10 μmol/L)+verapamil (1 μmol/L)+acetylcholine (ACh; 3 μmol/L) were given to the perfusate then temperature was lowered to 32°C and VT/VF developed. After the restorement of the temperature to physiologic, the arrhythmia disappeared (not shown). Cilostazol was capable to prevent arrhythmic events, ECG and action potential abnormalities after the reinduction of hypothermia.*[Gurabi et al. 2014]

	Endo APD90 (ms)	Epi1 APD90 (ms)	Epi2 APD90 (ms)	EDR (ms)	TDR (ms)	Notch Index*	Notch magnitude (% of Ph0)	Notch-duration (ms)	J wave area (mV × ms)
Control (37°C)	213.2 ±4.9	192.7 ±4.2	196.2 ±4.2	6.6 ±2.1	13.5 ±5.0	208.5 ±46.7	10.8 ±2.6	19.3 ±1.1	1.5 ±0.5
Provocative agents† (37°C)	230.5 ±6.9	202.4 ±8.5	211.0 ±9.0	5.9 ±3.3	15.0 ±7.4	944.2 ±132.9	29.2 ±2.1[d]	31.9 ±3.3	8.6 ±1.2
Provocative agents† (32°C)	345.1 ±26.0b	159.6 ±12.9	285.2 ±24.6	119.4 ±34.4[b]	157.8 ±33.4[b]	6397.9 ±897.4[b]	47.7 ±1.3[b]	133.1 ±15.0[b]	53.9 ±15.2[b]
+Cilostazol (10 μM) (32°C)	336.9± 34.5	323.5± 32.9[f]	328.4± 32.3	8.6± 3.5[f]	14.9± 11.1[f]	2455.5 ±92.5[f]	35.6 ±3.9[f]	67.5 ±5.2[f]	17.8 ±3.1[e]

Table 6. *Effects of hypothermia in the setting of ER on action potential duration at 90% of repolarization (APD90), maximum epicardial (Epi) dispersion of repolarization (EDR), transmural dispersion of repolarization (TDR), notch index, notch magnitude, notch duration and J wave area and the effects of quinidine on these parameters. *Notch index= Notch magnitude × (Ph 0 to Ph 2 interval) which approximates the area of the notch. †Provocative agents= NS5806 3-10 μM + Verapamil 1 μM + Acetylcholine 3 μM. Results are mean ± S.E.M. a=p<0.05,b=p<0.01 32°Celsius vs. 37°Celsius; c=p<0.05 NS5806 3-10 μM + Verapamil 1 μM + Acetylcholine 3 μM (37°Celsius) vs. Control (37°Celsius); e=p<0.05, f=p<0.01 Cilostazol 10 μM (32°Celsius) vs. NS5806 3-10 μM + Verapamil 1 μM + Acetylcholine 3 μM (32°Celsius).*[Gurabi et al. 2014].

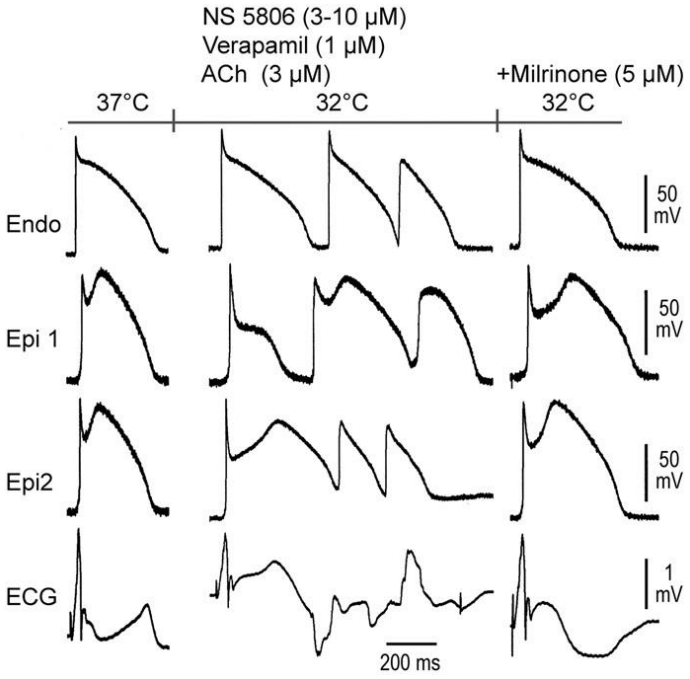

Figure 9. *Arrhythmogenic effect of hypothermia in the setting of early repolarization and the preventive effect of cilostazol. Action potential traces simultaneously recorded from 2 epicardial (Epi) and 1 endocardial (Endo) sites together with a pseudo-ECG. NS5806 (3–10 μmol/L)+verapamil (1 μmol/L)+acetylcholine (ACh; 3 μmol/L) were given to the perfusate then temperature was lowered to 32°C and VT/VF developed. After the restorement of the temperature to physiologic, the arrhythmia disappeared (not shown). Milrinone was capable to prevent arrhythmic events, ECG and action potential abnormalities after the reinduction of hypothermia.*[Gurabi et al. 2014]

	Endo APD90 (ms)	Epi1 APD90 (ms)	Epi2 APD90 (ms)	EDR (ms)	TDR (ms)	Notch Index*	Notch magnitude (% of Ph0)	Notch-duration (ms)	J wave area (mV × ms)
Control (37°C)	215.9 ±9.9	192.7 ±4.6	198.5 ±4.1	3.9± 1.3	9.7 ±1.6	230.0± 87.9	12.3 ±3.9	17.5 ±1.0	2.0 ±0.5
Provocative agents† (37°C)	243.5 ±7.2	203.3 ±6.4	210.8 ±2.1	9.3± 3.0	19.5 ±5.5	816.8± 119.3	26.1± 4.5[c]	32.1 ±1.5	8.1 ±0.8

Provocative agents† (32°C)	362.0 ± 35.8[b]	171.9 ± 29.6	353.5 ± 22.3[b]	175.3 ± 21.9[b]	170.9 ± 51.6[b]	5568.0 ± 1022.7[b]	49.6 ± 2.1[b]	109.8 ± 16.2[b]	56.3 ± 14.4[b]
+Milrinone (5 μM) (32°C)	371.2 ± 14.9	341.6 ± 22.5[f]	345.4 ± 16.7	16.8 ± 2.9[f]	18.2 ± 5.5[f]	2521.5 ± 578.3[f]	35.8 ± 3.2[f]	68.4 ± 10.5[e]	23.2 ± 7.7

Table 7. *Effects of hypothermia in the setting of ER on action potential duration at 90% of repolarization (APD90), maximum epicardial (Epi) dispersion of repolarization (EDR), transmural dispersion of repolarization (TDR), notch index, notch magnitude, notch duration and J wave area and the effects of Milrinone on these parameters. *Notch index= Notch magnitude × (Ph 0 to Ph 2 interval) which approximates the area of the notch. †Provocative agents= NS5806 3-10 μM + Verapamil 1 μM + Acetylcholine 3 μM. Results are mean ± S.E.M. a=p<0.05,b=p<0.01 32°Celsius vs. 37°Celsius; c=p<0.05 NS5806 3-10 μM + Verapamil 1 μM + Acetylcholine 3 μM (37°Celsius) vs. Control (37°Celsius); e=p<0.05, f=p<0.01 Milrinone 5 μM (32°Celsius) vs. NS5806 3-10 μM + Verapamil 1 μM + Acetylcholine 3 μM (32°Celsius* [Gurabi et al. 2014].

3.2 Effects of mexiletine enantiomers on transmembrane action potentials

The effects of 20 μM R-(-) and S-(+) enantiomers of mexiletine on action potential characteristics in rabbit ventricular/papillary muscle preparations are summarized in Table 8, with examples illustrated in Figure 10. At a stimulation cycle length of 1000 ms the enantiomers did not change ventricular repolarization (Table 8. and Figure 10.). Neither R- nor S-mexiletine had effects on the action potential amplitude, the maximal diastolic potential/membrane resting potential. Both R- and S-mexiletine significantly increased the ERP/APD_{90} ratio (Table 8.). Both enantiomers at 20 μM concentration significantly depressed the Vmax (Table 8.) at a stimulation cycle length of 1000 ms. The depression of maximal rate of rise of depolarization (Vmax) evoked by the stereoisomers (Figures 11A and 11B) was strongly dependent upon stimulation frequency ("use dependent"); i.e. as pacing cycle length was decreased (basic cycle lengths 300 ms – 700 ms), the depression of Vmax was increased. There was no statistically significant difference between mexiletine isomers in the degree of inhibition of Vmax (Figure 11C), though R-(-) mexiletine demonstrated a somewhat more potent inhibitory effect. Impulse conduction time (CT), another sodium channel-mediated parameter, i.e.: the time between the stimulus signal and the action potential upstroke was also prolonged significantly (Figures 12 A and B) by the enantiomers at fast rates. The effects of mexiletine enantiomers on early premature action potentials following final repolarization of the previous basic action potential (i.e., at a diastolic interval = 70 ms) are summarized in Table 9. Both R-(-) and S-(+) mexiletine significantly depressed the Vmax (Table 9.) of early premature action potentials and R-(-) mexiletine caused a more potent inhibitory action. At the stimulation cycle length of 1000 ms, mexiletine enantiomers inhibited the recovery of Vmax (Figure 13). The time constants for recovery of Vmax in the presence of 20 μM mexiletine enantiomers were $\tau = 376.0 \pm 77.8$ ms for R-(-) mexiletine and $\tau = 227.1 \pm 23.4$ ms for S-(+) mexiletine. The R-(-) mexiletine seems to display slower offset kinetics, i.e.: dissociation from the sodium channels, and the difference was significant ($p<0.05$) between mexiletine isomers in the degree of inhibition of recovery. The depression was predominant over the range of early extrasystoles.

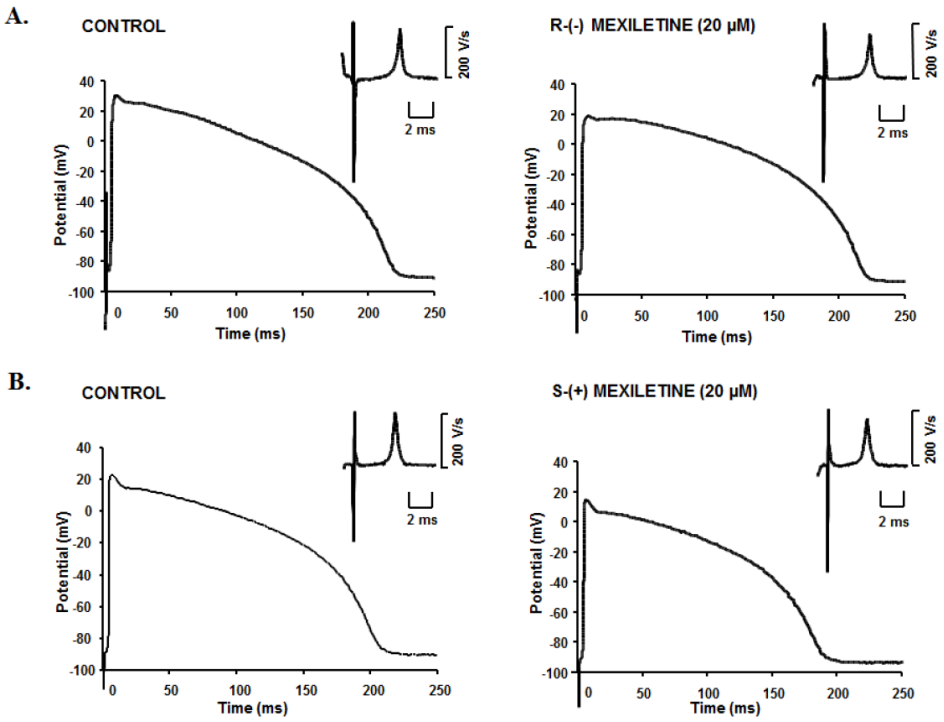

Figure 10. *Effects of R-(-)mexiletine (**A**) and S-(+) mexiletine (**B**) on action potential waveform of rabbit ventricular muscle at stimulation cycle length of 1000 ms. The amplitude and time scales for the recording of the first derivative of the transmembrane voltage with respect to time (Vmax) are indicated to the right.*

PARAMETERS	MDP (mV)	APA (mV)	APD_{90}(ms)	APD_{50} (ms)	Vmax (V/s)	ERP/APD_{90}
Control	-85.1 ± 1.2 (8)	108.7 ± 1.3 (8)	192.2 ± 8.4 (8)	156.0 ± 8.0 (8)	200.2 ± 13.1 (8)	0.953 ± 0.021 (6)
R-(-)Mexiletine 20 µM	-85.2 ± 1.7 (8)	104.2 ± 2.5 (8)	193.3 ± 6.9 (8)	155.7 ± 7.3 (8)	162.7 ± 7.9* (8)	1.031 ± 0.028* (6)
Control	-83.4 ± 1.7 (8)	105.9 ± 1.7 (8)	184.2 ± 11.9 (8)	148.9 ± 13.9 (8)	205.1 ± 12.3 (8)	0.942 ± 0.021 (6)
S -(+) Mexiletine 20 µM	-83.9 ± 1.1 (8)	106.9 ± 1.8 (8)	198.7 ± 13.1 (8)	161.3 ± 12.4 (8)	167.2 ± 10.5* (8)	0.997 ± 0.015* (6)

Table 8. *The electrophysiological effects of R -(-) and S-(+) mexiletine enantiomers in rabbit ventricular papillary muscles at basic cycle length of 1000 ms. Means ± SEM. MDP, maximum diastolic potential; APA, action potential amplitude; APD90, action potential duration at 90% of repolarization; APD50, action potential duration at 50% of*

repolarization; Vmax, maximum rising velocity of the action potential upstroke; ERP, effective refractory period; (n), number of observations (i.e., number of preparations obtained from different animals).

**p < 0.05*

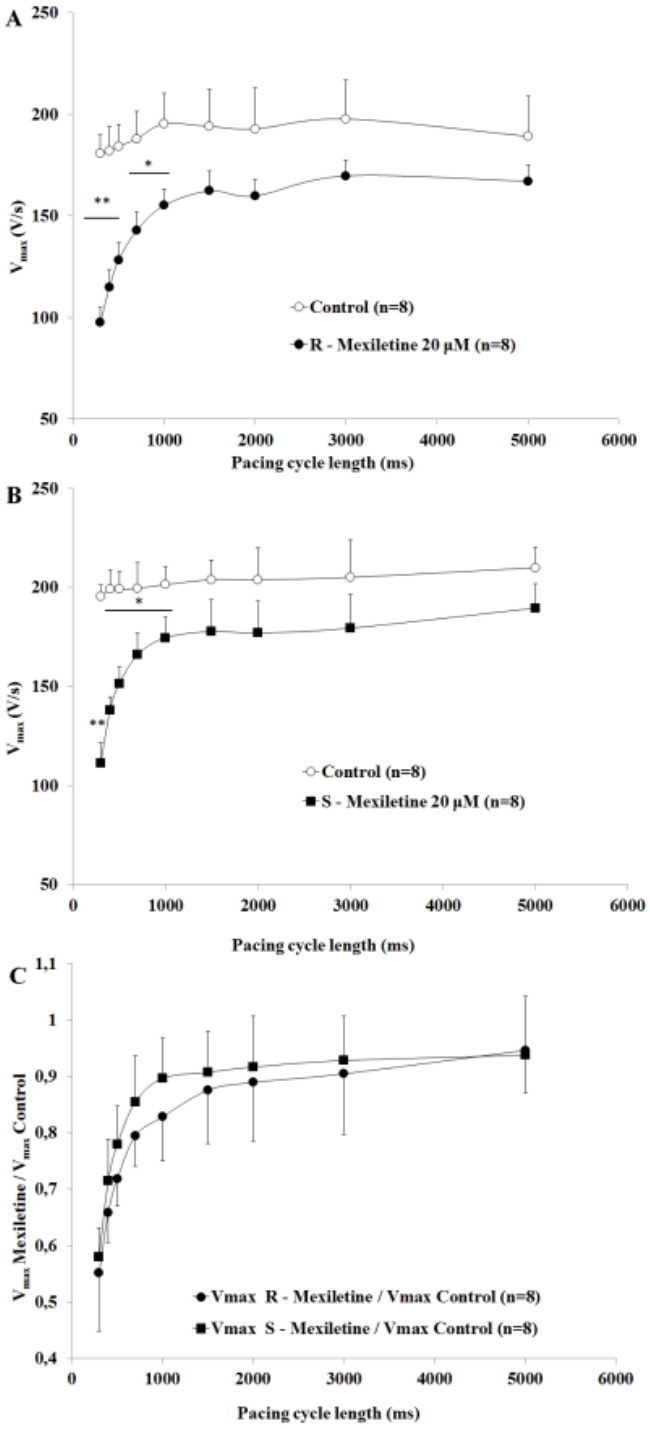

Figure 11. *Rate-dependent effects of R-(−) mexiletine (**A**) and S-(+) mexiletine (**B**) on the maximum rate of depolarization (Vmax). Results are mean ± SEM, *p < 0.05, **p < 0.01 vs. control, n = 8–8. Comparison of the rate-dependent effects of R-(-) mexiletine and S-(+) mexiletine on the maximum rate of depolarization (Vmax) (**C**). Values indicated on ordinate are calculated by dividing theVmax value measured in the presence of either R-(−) or S-(+) mexiletine with its own controlVmax value.*

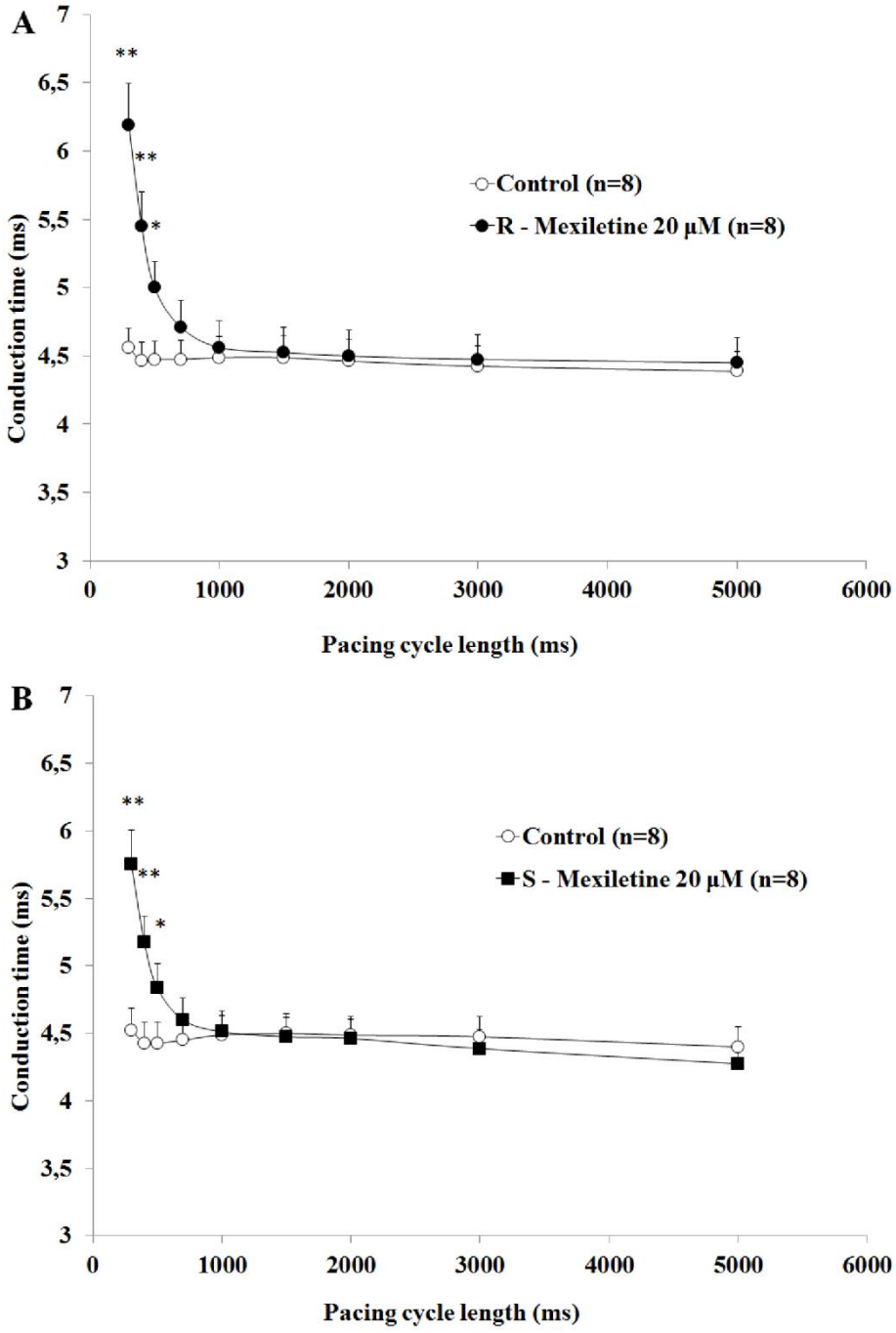

Figure 12. *Rate-dependent effects of R-(-) mexiletine (**A**) and S-(+) mexiletine (**B**) on the conduction time (CT). Results are mean ± SEM, *p < 0.05, **p < 0.01 vs. control, n = 8–8. The increase in CT in the presence of S-(+) isomer is less than that following exposure to the R-(−) isomer.*

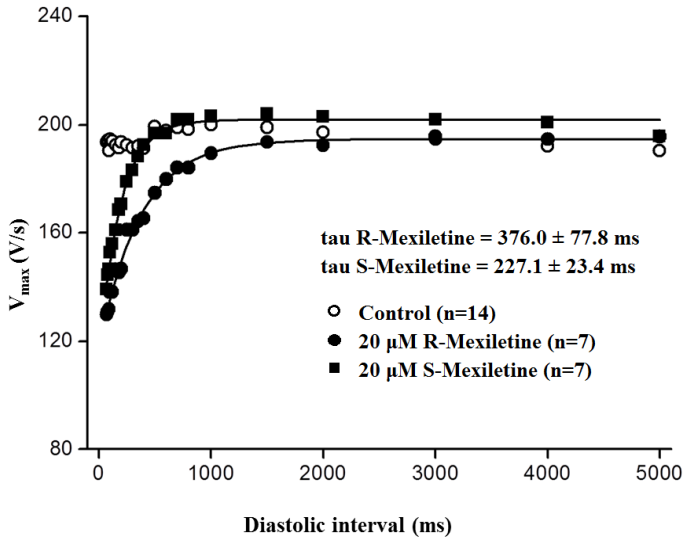

Figure 13.*Effects of R-(-) and S-(+) enantiomers of mexiletine on recovery of Vmax. Means are shown. The abscissa indicates diastolic intervals in milliseconds, and the ordinate shows theVmax. The time constants for recovery of Vmax were* τ *= 376.0 ± 77.8 ms for R-(-)mexiletine and* τ *= 227.1 ± 23.4 ms for S-(+) mexiletine. The difference was significant (p < 0.05) between mexiletine isomers in the degree of inhibition of recovery.*

PARAMETERS	MDP (mV)	APA (mV)	APD_{90}(ms)	APD_{50} (ms)	V_{max} (V/s)
Control	-83.4 ± 1.5 (7)	109.6 ± 2.1 (7)	204.4 ± 14.5 (7)	165.9 ± 15.7 (7)	208.2 ± 30.2 (7)
R-(-)Mexiletine 20 µM	-86.0 ± 0.9 (7)	107.9 ± 2.1 (7)	202.5 ± 9.5 (7)	161.5 ± 11.6 (7)	129.7 ± 29.9* (7)
Control	-85.0 ± 1.8 (7)	111.3± 2.6 (7)	200.7 ± 13.4 (7)	164.2 ± 13.9 (7)	185.6 ± 18.4 (7)
S -(+) Mexiletine 20 µM	-87.4 ± 1.2 (7)	110.0 ± 2.5 (7)	198.9 ± 18.5 (7)	162.0 ± 14.6 (7)	142.3 ± 13.6* (7)

Table 9. The electrophysiological effects of R-(−) and S-(+) mexiletine enantiomers on the premature action potentialat 70 ms of diastolic interval in rabbit ventricular papillary muscle. *Means ± SEM., MDP, maximum diastolic potential; APA, action potential amplitude; APD90, action potential duration at 90% of repolarization; APD50, action potential duration at 50% of repolarization; Vmax, maximum rising velocity of the action potential upstroke; (n), number of observations (i.e., number of preparations obtained from different animals).*p < 0.05*

4. DISCUSSION

4.1 Investigation of J-wave related arrhythmias in normo- and hypothermia and therapeutical possibilities

Mechanisms underlying early repolarization syndrome

Our study investigated the mechanisms underlying the development of electrocardiographic findings and arrhythmic manifestations of the early repolarization syndrome. We provided data to support the hypothesis that net outward shift in the balance of current in the early phases of action potentials in epicardium (due to I_{to} activation, I_{Ca} blockade and cholinergic antagonism) but not in endocardium is capable to accentuate the epicardial action potential notch, thus create a significant transmural voltage gradient between exaggerated epicardial action potential notch and intact endocardial action potential. These transmural voltage gradients and repolarization abnormalities resulted in J point elevation, distinct J waves, or slurring of the terminal part of the QRS. We pharmacologically modeled the genetic mutations in early repolarization syndrome by the addition of I_{to} agonist NS5806 and I_{Ca} antagonist verapamil. When this combination alone was unable to provoke – beside J point/wave elevation – loss of the action potential dome and a vulnerable window to VT/VF, we added ACh to mimic the increased vagal tone. Enhancement of parasympathetic influences in ER and its impact on J wave, ST-segment and on early repolarization syndrome (Marcus et al. 2002; Mizumaki et al. 2012) was described earlier; it was capable to enhance the already developed repolarization abnormalities (Gross et al 2010).

Parasympathetic influences on early repolarization pattern

In our experiments, we could create ER pattern, phase 2 reentry arrhythmia and VT/VF without the addition of acetylcholine, however when the I_{to} agonist NS5806 and I_{Ca} antagonist verapamil were not capable alone to create a loss of the dome AP morphology and phase 2 reentry, then acetylcholine was added and itcould provoke fibrillation. Parasympathetic activation might play a prominent role in the development of life-threatening arrhytmias in early repolarization syndrome (Koncz et al. 2014). Direct effects of acetylcholine were also investigated earlier, and acetylcholine seemed to accentuate the spike and dome morphology only in epicardial cells. Litovsky and Antzelevitch examined the direct effects of acetylcholine on endocardial and epicardial cells. Acetylcholine was found to have little if any effect on endocardial cells in the concentration of 10^{-7} – 10^{-5}μmol/L, however, it seemed to

influence action potential duration and effective refractory period in epicardium. It antagonized the isoproterenol-induced abbreviation of action potential duration and effective refractory period: this antagonizing effect of ACh was shown to be more pronounced in epicardium than in endocardium. In their study, all of the actions of acetylcholine could be reversed by atropin (Litovsky and Antzelevitch 1990). It was also demonstrated in canine heart preparations that parasympathetic activation exerts a significant negative inotropic effect on ventricular myocardium and reduced the left ventricular pressure. Supramaximal stimulation of nerves vagus caused a mean reduction of 23% in peak pressure (Degeest et al. 1965). Accentuated antagonism – an interaction between parasympathetic and sympathetic fibers of the heart – was investigated earlier. Parasympathetic activation seemed to antagonize the effects of sympathetic stimulation to increase I_{Ca}: when acetylcholine was infused alone into the dog coronary artery, it could provoke only a moderate decrease in myocardium contractility. However, the same infusion of acetylcholine – under the enhanced activation of sympathetic nervous system (due to infusion of norepinephrine) – was capable to create a much more depressed contractility (Hollenberg et al. 1965, Levy 1971). Another study (Levy and Zieske 1969) described similar effects in canine isovolumetric left ventricle preparations, where the depressant effect of vagal stimulation (on left ventricular systolic pressure) was much greater under sympathetic stimulation compared to baseline conditions (i.e. without sympathetic activation).

Effects of hypothermia under baseline conditions in the setting of early repolarization pattern

In our study under baseline conditions, hypothermia did not provoke any arrhythmia. Lowering the temperature to 32°C caused a prominent J wave enlargement in the ECG and accentuation of epicardial action potential notch but no arrhythmia appeared. These changes were reversible: after temperature was set back to physiologic (37°C), the ECG and action potential parameters normalized. When we used NS5806, verapamil and ACh in low concentrations at physiologic temperature, we could create an early repolarization pattern without arrhythmia. Under these conditions did not develop any arrhythmia but the phenotype of ER on the ECG. In the setting of early repolarization pattern, we demonstrated that the effect of mild hypothermia (32°C–34°C) can produce a prominent J wave, cause an all-or-none repolarization and make a loss of action potential dome morphology at some epicardial sites but not others, and make the action potential dome to propagate from regions at which it was maintained to regions at which it was lost. Nowadays, therapeutic hypothermia is a widely used therapeutic approach to prevent tissue injury after a myocardial infarction,

cardiac arrest, ischemic stroke, brain and spinal cord injury or neurogenic fever. Patients in these severe conditions were cooled to a body temperature 32 – 34° Celsius (Schwartz et al. 2012; Rolfast et al. 2012). Another study investigated the success rate of therapeutic hypothermia on neurologic recovery after cardiac arrest. Patients, who underwent therapeutic hypothermia after cardiac arrest, were compared with patients who received standard treatment with normothermia. 75 of 136 patients had a favorable neurologic outcome in hypothermia group compared with 54 of 137 in the normothermia group (Hypothermia after Cardiac Arrest Study Group. 2002). Beside beneficial outcomes, adverse effects of lowering the body temperature were reported, too. Bastiaenen et al. and Federman et al. reported case histories in which patients suffered ventricular fibrillation after the application of therapeutic hypothermia following a cardiac arrest or an intracranial haemorrhage. In both case histories, early repolarization pattern was known before the use of hypothermia (Bastiaenen et al. 2010; Federman et al. 2013).

Therapeutic possibilities in the prevention of hypothermia induced ventricular fibrilliation in the setting of ER

Our study also demonstrated the ability of quinidine, cilostazol and milrinone to preserve the action potential spike and dome morphology and to prevent the phase-2-reentry and VT/VF after the application of mild hypothermia in the presence of early repolarization pattern. All of the three agents act to restore AP dome by causing an inward shift in balance of current: quinidine via its inhibition of I_{to} and phosphodiesterase III inhibitors cilostazol and milrinone via the augmentation of I_{Ca} due to their action to increase cAMP. These actions suppressed arrhythmic activity that was seen during the application of hypothermia in the setting of ER. Quinidine was previously described to be effective in the treatment of recurrent ventricular fibrillation associated with early repolarization syndrome. Quinidine normalized not only J wave elevation but also suppressed arrhythmic events (Sacher et al. 2014). In another study, many antiarrhythmic drugs were tested but quinidine was the most effective. It could decrease the number of fibrillations from 33 ± 35 episodes to nil over a follow-up of 25 ± 18 months in patients with inferolateral early repolarization syndrome (Haïssaguerre et al. 2009). The phosphodiesterase III inhibitor cilostazol and milrinone were also tested whether it can normalize ECG/action potential findings and suppress arrhythmic activity. Earlier, a case report showed that cilostazol was capable to prevent VT/VF in a 64-year-old woman with early repolarization syndrome who experienced many ICD shocks due to ventricular fibrillation during sleep or early morning (Iguchi et al. 2013). In another study, patients were

treated with cilostazol and bepridil after the implantation of ICD. Cilostazol was given to decrease the numbers of ventricular fibrillation, bepridil was applied to relieve the cilostazol associated side effects, e.g. sinus tachycardia, palpitations. After the addition of cilostazol, 6 of 7 patients remaind free of ventricular fibrillation. In three patients, cilostazol administration was stopped for 2 days (on the basis of its antiplatelet effects) because of the replacement of the ICD generator. After the discontinuation of cilostazol, distinct J waves returned and ICD shocks were necessary again due to ventricular fibrillation. The readministration of cilostazol diminished ECG abnormalities (Shinohara et al. 2014).

4.2 Investigation of mexiletine enantiomers

Possible mechanism

In our study, we analyzed the electrophysiologic differences between S-(+) and R-(-) enantiomers of mexiletine. Mexiletine is a non-selective voltage-gated sodium channel blocker which belongs to the Class IB anti-arrhythmic medicines with similar antiarrhythmic characteristics to lidocaine with good oral availability and longer half-life (Vaughan Williams 1998). Nowadays, mexiletine is used as a racemic compound that contains both of the isomers. Campbell investigated the effects of mexiletine on the maximum rate of depolarization in guinea pigs. It was found that mexiletine had a greater impact on Vmax at fast rates (BCL 300ms) compared to other Class I antiarrhythmic drugs, e.g. disopyramide (Class IA) and encainide (Class IC) (Campbell 1983). Time constant of Vmax recovery after abrupt changes in cycle length was also investigated and compared to other Class I antiarrhythmic drugs. In the presence of racemic mexiletine and it was found to be 1.35 ± 0.2 s at BCL 500 in guinea pigs (Varró et al. 1985), however the cardiac electrophysiological effects of sole mexiletine entantiomers S-(+) and R-(-) on rabbit papillary muscles were not investigated yet. To this end, we observed the cardiophysiological effects of mexiletine enantiomers at the concentration of 20 μmol/L on rabbit ventricular preparations. To prove whether the R-(-) and S-(+) enantiomers had different electrophysiological properties, we investigated their effects on Vmax (the maximum rising velocity of the action potential upstroke) which is indicative for I_{Na} function. At first, both of the enantiomers were analyzed at a constant stimulation of basic cycle lenght 1000 ms. The enantiomers attenuated significantly Vmax and the *effective refractory period / APD90 ratio*, however, significant differences between enantiomers were not found. Then we changed pacing cycle length and we found that both of the enantiomers decreased significantly the Vmax between BCL 300 – 1000 ms (Figure 11 AB.), however, when we compared the *Vmax mexiletine / Vmax control*

ratio of both enantiomers (Figure 11 C.) the R-(-) enantiomer seemed to have a stronger inhibitory effect on Vmax. The comparison of the enantiomers by means of this *ratio* showed that R-(-) mexiletine made a more pronounced depression of Vmax at a cycle length range of 400–1000 ms than S-(+) mexiletine. The stronger depression of Vmax by the R-(-) enantiomer can be attributed to the slower offset kinetics compared to S-(+) enantiomer. Both of the enantiomers were capable to decrease Vmax of early premature action potentials significantly, however, in the case of R-(-) mexiletine we have seen a greater blocking effect on Vmax (Table 9). On these grounds, we assume that application of sole R-(-) mexiletine containing medications might decrease the probability of a second conducted impulse occurring at short premature intervals and thereby reduces the possibility of developing re-entrant tachyarrhythmias. Earlier, mexiletine stereoisomers were investigated on sodium channels of frog skeletal muscle. In the study of De Luca et al., the R-(-) mexiletine produced a tonic blockade of the sodium current of frog skeletal muscle with an IC50 of 43.9 ± 1 μmol/L, as long as S-(+) mexiletine was necessary at a twofold higher concentration to reach the same blocking effect (De Luca et al. 1995). Furthermore, another class I antiarrhythmic drugs (e.g lidocaine) seemed to be more effective in the inhibition of I_{Na} when the resting membrane potential is partly depolarized, e.g in ischeamic tissues (Pu et al. 1998). On this account, the effects of R-(-) mexiletine on Vmax could be further explored in future studies that apply cardiac preparations exposed to ischemia to see whether it could be beneficial in ischemic heart diseases. Besides Vmax measurements, we recorded another important parameter -the impulse conduction time (CT) - that can demonstrate the availability of sodium channels. We tested conduction time at different cycle length stimulations (300 – 5000 ms, Figure 12) before the addition of any compound, but conduction time remained unaltered. After the administration of R-(-) or S-(+) enantiomer, we have seen a marked significant prolongation at fast stimuli (BCL 300 – 500 ms) by each enantiomers compared to baseline conditions, however, R-(-) mexiletine was capable to produce a slightly longer prolongation. The difference between the two enantiomers was not significant in the point of conduction time. Finally, the time constant for recovery of Vmax was analised (Figure 13) and R-(-) mexiletine displayed a slower offset kinetics than S-(+) mexiletine, which means that R-(-) enantiomer is capable to decrease Vmax more effectively in later developed extrasystoles than S-(+).

Clinical implications

A recent study highlighted the benefical effects of mexiletine in patients with an implantable cardioverter defibrillator. By the examined patients, amiodarone acted ineffectively, therefore the therapy was augmented with mexiletine. Subjects with combined – amiodarone and mexiletine – therapy performed reduced numbers of ventricular tachycardia/fibrillation compared to a matched duration of observation just before initiating mexiletine (Gao D. et al 2013). Another study observed patients with Long-QT syndrome type 3; oral mexiletine was administered at a duration of 36 months, and the results were compared to the time period before mexiletine therapy. Mexiletine was found to be effective in the reduction of *the percentage of patients with arrhythmic events*; *the mean number of arrhythmic events per patients* and *the annual rate of arrhythmic events*. On the ECG recordings, a significantly shortened QTc were seen (Mazzanti et al. 2016). In another study, the role of mexiletine were investigated in patients with torsades de pointes on the basis of acquired long QT syndromes. Mexiletine was used after conventional treatment options (e.g discontinuation of QT-prolonging drugs, intravenous magnesium and correction of serum electrolyte abnormalities) failed to work. Two hours after the addition of mexiletine, no TdP arrhythmia appeared again. On the ECG recordings, shortening of the QTc interval, reduction of T-wave$_{peak\text{-}end}$ interval and a decrease of T-wave$_{peak\text{-}end}$/QT ratio were seen without significant effect on QRS complex duration (Badri et al. 2015). Sicouri et al. investigated the molecular background of the therapeutical effect of mexiletine in LQTS. They used the I_{Kr} blocker d-sotalol to mimic the HERG defect in LQTS2 and anemone toxin (ATX-II) to mimic the SCN5A defect in LQTS3. Both agents prolonged the action potential durations, d-Sotalol in mid-myocardial M cells, while ATX-II caused APD90 prolongation in endocardial, epicardial and mid-myocardial cells. Mexiletine could reverse d-sotalol and ATX-II produced prolongation in mid-myocardial cells, however, it failed to reverse significantly the epicardial and endocardial actions of ATX-II. Due to its actions on M-cells, it was capable to reduce transmural dispersion of repolarization and it suppressed successfully early afterdepolarization and early afterdepolarization induced triggered activity (Sicouri et al. 1997). Shimizu and Antzelevitch observed the effectiveness of mexiletine in the same LQTS2 and LQTS3 modell. Mexiletine (2-20 μmol/L) dose-dependently shortened the drug induced QT-, APD90- lengthening and decreased TDR. In a concentration of 2–5 μmol/L, it suppressed spontaneous torsade de pointes (TdP). In higher concentration (10–20 μmol/L), it totally suppressed stimulation-induced TdP (Shimizu and Antzelevitch 1997). Mexiletine enantiomers were investigated earlier in experimentally-induced arrhythmia dog-models. Ventricular fibrillations were

provoked by programmed electrical stimulation 30 days after coronary ligation. R-(-) mexiletine proved to be more effective as it was capable to prevent arrhythmias in 3 out of 6 dogs while S-(+) mexiletine was capable to protect against arrhythmia in 1 ouf of 5 dogs. Their work showed an evidence that R-(-) mexiletine may possess stronger antiarrhythmic characteristics than the opposite enantiomer (Turgeon et al. 1991). On the basis of the findings of De Luca (greater inhibitory potency of R-(-) mexiletine on I_{Na} in frog skeletal muscle) and Turgeon (more effective protection against ventricular fibrillation) and our findings, it might be worth exploring the impacts of the mexiletine enantiomers,but especially those of the R-(-) mexiletine in different patientgroups, i.e, patients with an implantable cardioverter defibrillator and under amiodarone therapy or in LQT3 or LQT2 patients. Varró and Lathrop figured out from Purkinje fiber experiments that the simultaneous administration of sotalol and mexiletine could produce a beneficial electrophysiological effect by intensifying the therapeutical antiarrhythmic effect as long as proarrhythmic effects could be minorated (Varró and Lathrop 1990); therefore, it is worth the effort to explore the combined effects of sotalol and R-(-) mexiletine in future experiments and clinical studies. The pharmacokinetic properties of both enantiomers were investigated earlier and compared with each other. Kwok et al. investigated 12 healthy human subjects 6 hours after the oral administration of 200 mg racemic mexiletine: the mean peak serum total mexiletine concentration for R-(-) mexiletine (217 ± 69 ng/ml for R-(-) vs 197 ± 56 ng/ml for S-(+)mexiletine) and mean serum total R-(-) mexiletine concentrations were found to be significantly higher compared to S-(+) mexiletine (Kwok et al. 1995). McErlane et al. studied the differences between the protein binding features of mexiletine enantiomers using a stereoselective high-performance liquid chromatographic method involving 5 healthy human male subjects. Racemic mexiletine was added to reach a concentration range 0.2 to 2.0 μg/ml. They found that R-(-) mexiletine showed a significantly greater binding tendency to serum proteins. Free fraction of S-(+) enantiomer was 28.32 ± 1.45%, while in the case of R-(-) enantiomer it was 19.80 ± 1.49% (McErlane et al. 1987). Another study examined the pharmacokinetics of mexiletine enantiomers by using the *R-(-) mexiletine / S-(+) mexiletine concentration ratio* (R/S). R/S concentration ratio in plasma was 1.37 ± 0.11 after 1 hour and 0.64 ± 0.11 two days after the administration of 300 mg single oral dose of racemic mexiletine. Analogical results were found in urine concentration, where R/S concentration ratio was 1.38 ± 0.42 after 1 hour and 0.55 ± 0.12 after 72 hours. Terminal elimination half-life of S-(+) mexiletine was significantly greater compared to R-(-) mexiletine (11.0 +/- 3.80 hours vs 9.10 +/- 2.90 hours) (Igwemezie et al. 1989). A study in rats (Mehvar et al. 2002)

uncovered non-stereoselective distribution of the isomers to most tissues with the exception of the liver, where the concentration of the S-(+) stereoisomer was more than twice of that of its antipode. This study also established a 2-fold higher tissue/serum concentration ratio for S-(+) mexiletine enantiomer as compared with the ratio for the R-(–) isomer. The most frequently seen adverse effects of mexiletine are often dose related and they affect mainly the central nervous system. Tremors, nystagmus, blurred vision, dizziness, drowsiness, confusion, ataxia, paresthesia, dysarthria, insomnia, tinnitus, and convulsions are the most common side effects. Gastrointestinal disturbances can also develop during mexiletine therapy (Vaughan Williams 1998). Detailed studies are still needed to establish the pharmacokinetic difference between the 2 stereoisomers and its possible therapeutic and toxicological significance.

5. CONCLUSION

Our study demonstrated the effect of hypothermia to accentuate repolarization abnormalities within the left ventricular epicardium and that in the setting of ER, this effect of hypothermia is accentuated leading to the development of phase 2 reentry and VT/VF. We also provided support for the hypothesis that agents capable of producing an inward shift in the balance of current during the early phases of the AP can exert a protective and/or ameliorative effect. Quinidine by virtue of its Ito inhibition and cilostazol and milrinone, by virtue of their effects to augment ICa, were found to be effective in partially reversing the hypothermia-induced repolarization abnormalities, thus restoring electrical homogeneity and abolishing the arrhythmogenic substrate.

Our investigations with the mexiletine enantiomers resulted in the following conclusions to be withdrawn: slower detachment kinetics of R-(-) mexiletine from the sodium channels than that of S-(+) mexiletine and pronounced suppression the Vmax of early extrasystoles by R-(-) mexiletine might be of therapeutic value. Using lower doses of the probably more potent R-(-) mexiletine in the therapy of different disease conditions (arrhythmias, abnormal hyperexcitability of the myotonic muscles, neuropathic pain, ALS), might result in the reduction of unwanted adverse effects mentioned. To establish, whether the application of R-(-) mexiletine alone would result in enhanced antiarrhythmic efficacy requires further in vitro and an vivo studies.

Study Limitations

As with all data derived from experimental animal models, extrapolation of the data from in vitro models to the clinic must be done with great care.

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