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Jia, L.

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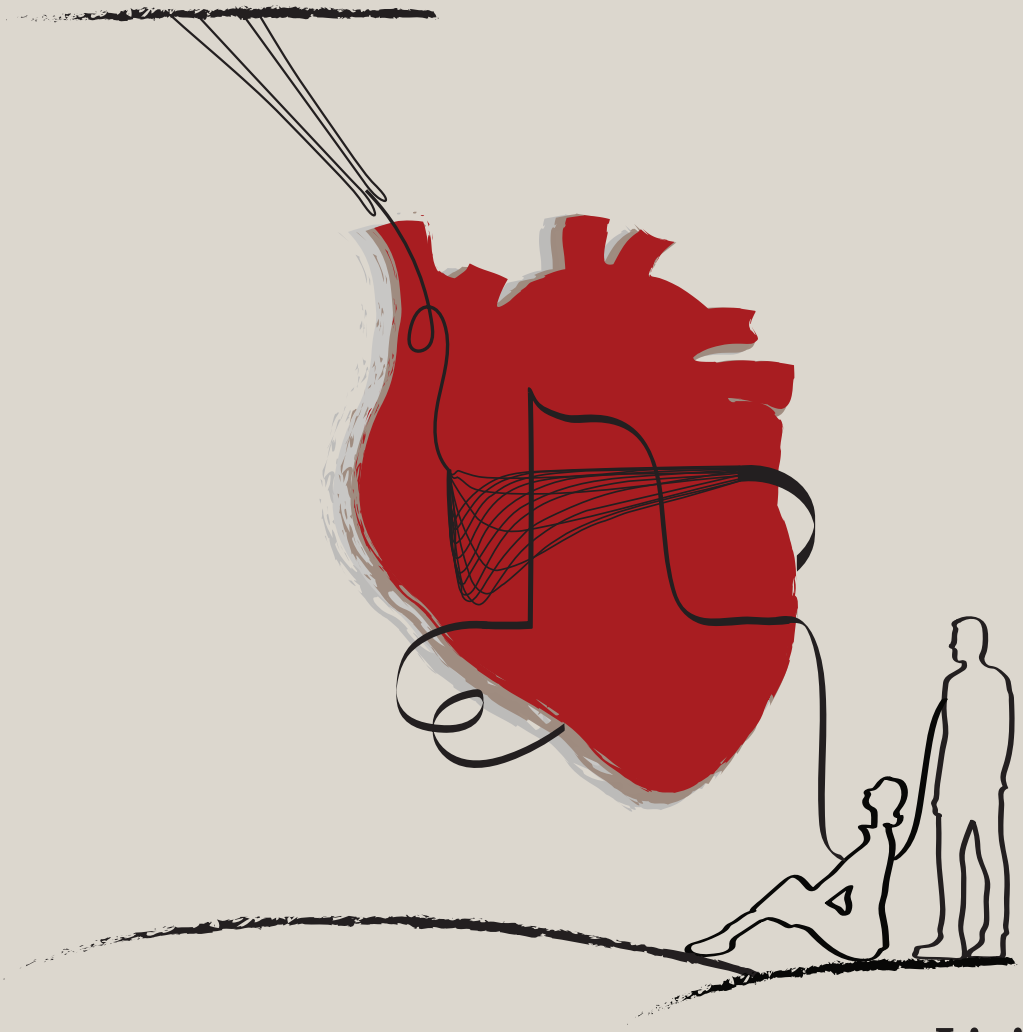

Lixia Jia
贾丽霞

CARDIAC ION CHANNEL DYSFUNCTION UNDERLYING SUDDEN CARDIAC ARREST ASSOCIATED WITH NONCARDIAC DRUGS

Lixia Jia

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Cardiac ion channel dysfunction underlying sudden cardiac arrest associated with noncardiac drugs

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CHAPTER 1

INTRODUCTION AND OUTLINE

1 OVERVIEW

Sudden cardiac arrest (SCA) is the sudden cessation of the heart's ability to pump blood through the body. SCA accounts for 20% of overall mortality and 50-60% of cardiovascular mortality in industrialized societies, making it a major public health challenge.[1-3] The immediate cause of SCD in most instances is ventricular arrhythmia - either ventricular fibrillation (VF) or ventricular tachycardia (VT). VF/VT may be secondary to changes in the function of the cardiac sarcolemmal ion channels that control the heart's electrophysiological properties.[4, 5] Current evidence has identified an increasing number of drugs that may impact on the risk of the occurrence of VF/VT, and SCA.[6-10] The major mechanism whereby these drugs induce proarrhythmic effects is their effect on cardiac ion channel function.[11, 12] Drugs that impair cardiac repolarization (QT-prolonging drugs) or cardiac depolarization (sodium [Na^+] channel-blocking drugs) are two major groups of drugs that have been implicated in this proarrhythmic category.[13, 14] The Survival With Oral D-sotalol (SWORD) study demonstrated that cardiac antiarrhythmic drugs can increase SCA risk by blocking the rapid component of the delayed rectifier potassium [K^+] current (I_{Kr}).[15, 16] Similarly, the Cardiac Arrhythmia Suppression Trial (CAST) revealed that cardiac antiarrhythmic drugs can increase SCA risk by blocking the cardiac Na^+ current.(I_{Na}) [17]

Apart from these cardiac antiarrhythmic drugs whose therapeutic action depends on block of I_{Kr} (Vaughan-Williams class 3 or 1a drugs) or I_{Na} (Vaughan-Williams class 1c drugs and amiodarone), several noncardiac drugs (drugs prescribed for the treatment of noncardiac disease) also impact on VF/SCA risk because they interact with cardiac ion channels such as I_{Kr} and I_{Na} (as an off-target effect), typically blocking them and thereby increasing VF/SCA risk. This group of drugs is extensive and includes drugs prescribed for a variety of conditions, including anti-epileptic drugs, opioids, antidepressants, antipsychotics, antibiotics, antifungals, antihistamines, and oncolytics. Clinically, this is relevant, because these drugs are typically prescribed by non-cardiologists who have limited awareness of the proarrhythmic potential of these drugs and/or have few means to evaluate their patients' risk when prescription of these drugs is considered and/or to monitor the risk once these drugs are prescribed. Accordingly, on a general population level, the SCA risk associated with noncardiac QT-prolonging drugs is paradoxically larger than that of cardiac QT prolonging drugs.[18]

Clearly, it is vital to recognize in time that a particular drug blocks cardiac ion channels as an off-target effect and may thereby increase SCA risk, because the timely identification of such drugs may lead to start of timely measures to prevent drug-induced SCA. General treatment guidelines focus on avoidance of prescription of these drugs to vulnerable individuals. Such vulnerability may be based on the presence of comorbidities linked with diminished ion channel function.[19] Importantly, different comorbidities may impact ion channels differently. Often, one type of ion channel is affected predominantly. For instance, myocardial ischemia and infarction are predominantly associated with I_{Na} reduction. Consistent with this change, excess

SCA incidence among study participants who were randomized to class 1c antiarrhythmic drugs in the CAST trial occurred in patients with active myocardial ischemia.[17] Conversely, the predominant electrophysiologic effect of heart failure is reduction in cardiac repolarizing currents. Accordingly, excess mortality among study participants who were randomized to class 3 antiarrhythmic drugs in the SWORD trial occurred in patients with heart failure who had a left ventricular ejection fraction of 31-40%.[15] Thus, knowledge about the electrophysiological mechanism whereby a drug increases SCA risk (by blocking I_{Na} or I_{Kr} or by impacting on another ion channel) is important to design rational prevention strategies to avoid drug-induced SCA. For instance, drugs which block I_{Na} may have to be avoided (or used only after taking appropriate precautions) by patients with myocardial ischemia, while drugs which block I_{Kr} may have to be avoided by patients with heart failure. In this thesis, we explored the possible mechanism whereby anti-epileptic drugs (AEDs) and opioids increase the risk of SCA, and sulfonylurea antidiabetics decrease the risk of SCA, on a cellular level.

2 ION CHANNEL DYSFUNCTION AND SUDDEN CARDIAC ARREST

2.1 Cardiac action potential

Action potentials (APs) are produced by the coordinated action of transmembrane ion channels in individual cardiac cells. The generation of APs, which consist of five phases, requires a series of ion movements across the cell membrane to transfer the cell from its resting state to its activated state (depolarization) and back to its resting state (repolarization).[20] In ventricular myocytes, when a positive change of transmembrane voltage reaches a threshold value (around –60 mV), APs are triggered by the acute and large influx of Na^+ ions into the cell, resulting in an inward current (I_{Na}) that shifts the membrane potential from its resting state to a depolarized state.[21] This is phase 0, which is also called upstroke or rapid depolarization. The equation dV/dt_{max} or Vmax indicates the maximal rate of depolarization that takes place during phase 0, and is similar to the maximum rate of change in voltage over time. Following phase 0, the efflux of K^+ ions through an outward current named transient outward K^+ current (I_{to}), initiates cell repolarization, which is called early rapid repolarization (phase 1). Next comes the AP plateau phase (phase 2), a period of constant membrane potential due to the balance between inward Ca^{2+} currents ($I_{Ca,L}$) through the L-type voltage-dependent Ca^{2+} channels and outward K^+ currents through the slow component of the delayed rectifier K^+ current (I_{Ks}) and I_{Kr}. During phase 3 (rapid repolarization phase), the Ca^{2+} channels close due to inactivation, while the K^+ channels continue to conduct K^+ ion out of the cell; in this way, the electronegative membrane potential is restored. Phase 4 is the phase between two APs with a resting membrane potential (RMP) of around –90 mV under normal physiological conditions in ventricular myocytes, and this phase is importantly set by the inward rectifier K^+ current (I_{K1}). The above-mentioned ion channels are the main channels involved in the cardiac AP, but additional currents, such as the ATP-regulated K^+ channel ($I_{K\text{-}ATP}$) also contribute to the AP profile under specific

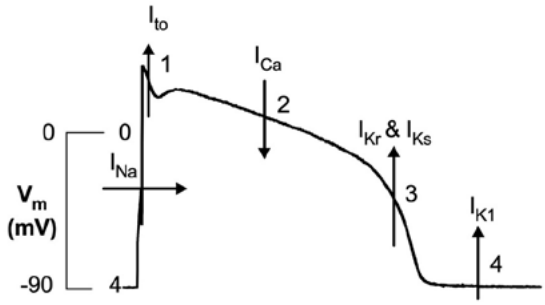

Figure 1. Action potential (AP) waveform and underlying ionic currents in ventricular myocytes. The numbers refer to the 5 different APs phases as explained in the above accompanied text. V_m = membrane potential, I_{Na} = Na^+ current, I_{to} = transient outward K^+ current, I_{Ca} = Ca^{2+} current, I_{Kr} and I_{Ks} = rapid and slow components of the delayed rectifier K^+ currents, I_{K1} = inward rectifier K^+ current.

Table 1. The main ion currents, and the corresponding channel proteins and the genes that encode them, which contribute to the ventricular action potential.

Current name	α subunit protein	gene	phase
I_{Na}	Nav1.5	*SCN5A*	0
I_{to}	Kv1.4, Kv4.3	*KCNA4, KCND3*	1
$I_{Ca,L}$	Cav1.2	*CACNA1C*	1, 2
I_{Ks}	KvLQT1	*KCNQ1*	2,3
I_{Kr}	HERG	*KCNH2*	2,3
I_{K1}	Kir2	*KCNJ2, KCNJ12*	3, 4

For abbreviations, see Figure 1.

situations such as myocardial ischaemia/infarction.[22] This thesis focuses on three ion currents: I_{Na}, I_{Kr}, and $I_{K\text{-}ATP}$.

2.2 Noncardiac drugs that block Nav1.5 channels

SCN5A is the gene encoding the a-subunit of the cardiac Na^+ channel (Nav1.5) whose current, I_{Na}, is responsible for phase 0 of the cardiac AP.[23] *SCN5A* loss-of-function mutations leading to dysfunctional or fewer Nav1.5 channels at the cellular membrane may provoke decreased excitability and slow cardiac conduction. For instance, the reduction of I_{Na} plays a central role in the mechanism of Brugada Syndrome (BrS),[24, 25] which has been recognized as an important cause of SCA in young individuals.[26, 27] *SCN5A* is the most prevalent gene involved in BrS, with 20–30% of BrS patients carrying loss-of-function mutations in this gene.[28] BrS is associated with a characteristic ECG pattern, termed the BrS-ECG.[29] In recent years, more and more noncardiac drugs have been recognized to have the potential to induce the BrS-ECG, signalling that these drugs may block I_{Na}.[30, 31] These drugs are listed on the website www.brugadadrugs.org,[31] which is often consulted by clinicians to assess whether drugs must be withheld from patients diagnosed

with BrS (in whom these drugs would cause excessive I_{Na}, leading to SCA). However, this website has clear limitations, because it is mostly based on anecdotal reports, while a systematic (biophysical) analysis of the proarrhythmic effects of most listed drugs and knowledge of their risk in the general population are lacking.[31] Thus, the risk of VF/SCA may be overestimated for some drugs, and underestimated (or unknown) for others. Anesthetics and psychotropic drugs are the most common non-cardiac drugs involved in drug-induced BrS.[31] The mechanisms of action of anesthetics and psychotropic drugs are related to modulating the neuronal Na^+ channels.[32-35] For instance, opioids such as tramadol and fentanyl could block neuronal I_{Na}.[36-38] Neuronal ion channels and cardiac ion channels share similarities in sequence and biophysical characteristics.[39, 40] This suggests a very high possibility that these drugs also block Nav1.5 channels and disturb normal cardiac electrophysiological activity. Yet, drug-induced Nav1.5 blockade has received far less attention than drug-induced cardiac K^+ current blockade. In this thesis, the impact of the six most often prescribed anti-epileptic drugs (AEDs) in the Netherlands (carbamazepine, gabapentin, lamotrigine, levetiracetam, pregabalin, and valproic acid) and the three most commonly prescribed opioids (tramadol, codeine, and fentanyl) on Nav1.5 current was tested to investigate if these noncardiac drugs block Nav1.5 current.

2.3 Noncardiac drugs that block repolarizing ion channels

The best-known category of drugs that may induce fatal cardiac arrhythmias are drugs which delay cardiac repolarization (QT prolonging drugs).[41, 42] The long QT syndrome (LQTS) is characterized by a long QT interval on the ECG and an increased risk of SCA.[43] Congenital LQTS is due to mutations in genes that encode cardiac ion channels which modulate the duration of the AP.[43] For instance, loss-of-function mutations in *KCNQ1* and *KCNH2* cause LQTS1 and LQTS2, respectively, by a decrease of repolarizing K^+ currents; conversely, gain-of-function mutation of *SCN5A* could induce LQTS 3 by an increase of a persistent component of the depolarizing Na^+ current. Compared to inherited LQTS, acquired LQTS, in particular, drug-induced LQTS, is more common. The main mechanism underlying drug-induced LQTS is also related to effects on transmembrane ion channels, in particular, K^+ channel blockade.[47] This was first discovered for quinidine. This drug was found to block I_{Kr}, thereby prolonging the AP duration (APD) and causing fatal ventricular arrhythmias, in particular, torsades de pointes (TdP).[48, 49] Drug-induced TdP was the most frequent reason for the withdrawal or restriction of the use of medicines that had already been approved for sale in the last decade.[8] Afterwards, more drugs were found to have similar effects. These drugs are listed on the website https://crediblemeds.org/ In this thesis, the possible effect of tramadol to cause AP prolongation was investigated, because various opioids were shown to block cardiac hERG channels (which underlie I_{Kr}) and induce LQTS and TdP, e.g., methadone.[50]

3 REDUCTION OF SCA RISK BY NONCARDIAC DRUGS

While most attention in the literature has been given to the possibility that noncardiac drugs increase SCA risk by blocking cardiac ion channels (as an off-target effect), it is conceivable that

effects of noncardiac drugs on cardiac ion channels may also act to reduce SCA risk. Noncardiac drugs can reduce the risk of SCA by affecting the upstream events including interactions between substrate, triggers and modulating factors,[51] e.g., angiotensin-converting enzyme inhibitors may reduce the incidence of hypokalaemia or inhibit ventricular remodelling in congestive heart failure,[52, 53] thereby reducing the risk of the occurrence of ventricular arrhythmias. In addition, noncardiac drugs may reduce SCA risk by interacting directly with cardiac ion channels. A possible cardiac ion channel target is the K_{ATP} channel that produces $I_{K\text{-}ATP}$. This channel becomes activated during acute myocardial ischaemia and infarction.[54, 55] Under physiological conditions, cardiac K_{ATP} channels are assumed to be mostly closed and contribute little to cell excitability.[54] However, the K_{ATP} channels are opened when the heart is subjected to reduced intracellular ATP levels, such as in acute myocardial ischemia and infarction.[55, 56] Acute myocardial ischemia and infarction are major immediate causes of SCA, accounting for around 80% of SCA cases.[5, 57] K_{ATP} channel activation speeds up cardiac repolarization, thereby causing APD shortening.[20] This is a potentially proarrhythmic effect, because it facilitates reentrant excitation and the occurrence of fatal arrhythmias.[58] This is illustrated by the Short QT syndrome in which gain-of-function mutations in KCNH2 produce I_{Kr} channels that cause QT shortening and a high risk of SCA.[59] Conversely, cardiac K_{ATP} channel blockers may reduce the risk of VF and SCA by counteracting APD shortening during acute myocardial ischemia and infarction.[60, 61] Sulfonylurea antidiabetics (e.g., glibenclamide, gliclazide, glimepiride, tolbutamide) are blockers of K_{ATP} channels. We studied whether these drugs reduce SCA risk and, if so, whether they prevented APD shortening during simulated ischemia in **chapter 5**.

4 OUTLINE OF THIS THESIS

In this thesis, I studied the effects on cardiac ion channels of three often used categories of noncardiac drugs: AEDs (chapters 2 and 3), opioids (chapter 4), and sulfonylurea antidiabetics (chapter 5). I mostly studied the possible effects of these drugs on I_{Na}, I_{Kr}, and $I_{K\text{-}ATP}$. In **chapter 2**, using rabbit and human cardiomyocytes, I explored the possible cardiac cellular electrophysiological effects of the AED carbamazepine and investigated the effect of carbamazepine on SCA incidence in the general population. In **chapter 3,** I studied the effects of other commonly prescribed AEDs - gabapentin, lamotrigine, levetiracetam, pregabalin, and valproic acid - on Nav1.5 current in HEK293 cells and investigated the effect of these drugs on rabbit ventricular AP properties. In **chapter 4,** I studied the effects of three often used opioids (tramadol, codeine, and fentanyl) on Nav1.5 current and rabbit ventricular APs. In chapter 5, I studied the cardiac effects of sulfonylurea antidiabetics. I investigated whether these drugs impact on SCA risk in the general population, and explored if these effects are related to changes in AP duration during simulated ischemia in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) in **chapter 5**.

The final chapter of this thesis (chapter 6) gives a general discussion of the topics mentioned above and addresses future views.

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CHAPTER 2

CARBAMAZEPINE INCREASES THE RISK OF SUDDEN CARDIAC ARREST BY A REDUCTION OF THE CARDIAC SODIUM CURRENT

Lixia Jia*, Talip E. Eroglu*, Ronald Wilders, Arie O. Verkerk, Hanno L. Tan

* Both authors contributed equally

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ABSTRACT

Aim

To assess the risk of sudden cardiac arrest (SCA) associated with the use of carbamazepine (CBZ) and establish the possible underlying cellular electrophysiological mechanisms.

Methods

The SCA risk association with CBZ was studied in general population cohorts using a case–control design (n = 5,473 SCA cases, 21,866 non-SCA controls). Effects of 1–100 μM CBZ on action potentials (APs) and individual membrane currents were determined in isolated rabbit and human cardiomyocytes using the patch clamp technique.

Results

CBZ use was associated with increased risk of SCA compared with no use (adjusted odds ratio 1.90 [95% confidence interval: 1.12–3.24]). CBZ reduced the AP upstroke velocity of rabbit and human cardiomyocytes, without prominent changes in other AP parameters. The reduction occurred at ≥30 μM and was frequency-dependent with a more pronounced reduction at high stimulus frequencies. The cardiac sodium current (I_{Na}) was reduced at ≥30 μM; this was accompanied by a hyperpolarizing shift in the voltage-dependency of inactivation. The recovery from inactivation was slower, which is consistent with the more pronounced AP upstroke velocity reduction at high stimulus frequencies. The main cardiac K^+ and Ca^{2+} currents were unaffected, except reduction of L-type Ca^{2+} current by 100 μM CBZ.

Conclusion

CBZ use is associated with an increased risk of SCA in the general population. At concentrations of 30 μM and above, CBZ reduces AP upstroke velocity and I_{Na} in cardiomyocytes. Since the concentration of 30 μM is well within the therapeutic range (20–40 μM), we conclude that CBZ increases the risk of SCA by a reduction of the cardiac I_{Na}.

Keywords: anti-epileptic drugs, sudden cardiac arrest, risk association, cardiomyocytes, sodium current, action potentials

1 INTRODUCTION

Sudden cardiac arrest (SCA) is a global public health problem with an annual incidence of 40–100 per 100,000 individuals[1, 2]. SCA accounts for 50% of deaths from cardiovascular disease and 15–20% of all deaths in industrialized societies.[3, 4] Most cases of SCA are caused by cardiac arrhythmias (ventricular fibrillation (VF) or ventricular tachycardia (VT)). Such arrhythmias may arise from functional changes in the ion channels that underlie the cardiac action potential (AP).[5] These functional changes may be evoked by various drugs used for the treatment of cardiac or non-cardiac conditions. This is best known for drugs that affect cardiac repolarization (QT prolonging drugs).[6] However, there is increasing recognition that it also applies to drugs that affect cardiac depolarization.[7] An example of such drugs are anti-epileptic drugs (AEDs).[8] Some AEDs are primarily developed for blocking neuronal ion channels, e.g., voltage-gated Na^+, Ca^{2+} or K^+ channels, while other AEDs act by impacting on neurotransmitters such as γ-aminobutyric acid.[9, 10] Importantly, neuronal and cardiac ion channel isoforms are highly homologous.[11, 12] Thus, AEDs may not only affect neuronal electrical activity but may also act on cardiac ion channels, thereby causing cardiac arrhythmias.[13] Accordingly, the increased SCA risk of epilepsy patients may be partly explained by AED use.[8]

Carbamazepine (CBZ) is a prime example of such drugs, because it has high efficacy in the treatment of epilepsy[14] through various mechanism, including block of neuronal Na^+ channels.[15-18] CBZ may also impact on cardiac electrophysiology as suggested by several CBZ-related case reports and retrospective studies, which report bradycardia, sinoatrial and atrioventricular block, QRS interval prolongation, cardiac arrhythmias, and cardiac arrest, as summarized in Table 1.[19-28] Still, the underlying electrophysiological mechanism is not completely understood. Our current study has two aims: 1) to establish whether CBZ is associated with increased SCA risk in a large dataset from a cohort that was specifically designed to study SCA in the general population; 2) to establish the effects of CBZ on cardiac APs and individual membrane currents of rabbit and human cardiomyocytes using patch clamp methodology.

2 METHODS AND MATERIALS

2.1 Epidemiological Studies

We studied the SCA risk associated with CBZ use in a case–control design. Cases were patients who suffered out-of-hospital SCA with presumed cardiac causes in the Amsterdam Resuscitation Studies (ARREST) registry. ARREST is an ongoing, prospective, population-based registry that we designed to study the occurrence and outcome of out-of-hospital SCA in the general population. Patients are collected in collaboration with dispatch centers, ambulance personnel, pharmacies and hospitals in one contiguous study region in the Netherlands (2.6 million inhabitants, urban and rural areas), thereby assuring collection of >95% of all out-of-hospital SCA patients in the study region and minimizing inclusion bias.[29] Each out-of-hospital SCA case was matched with up to five non-SCA controls based on age, sex and index-date (SCA-date). Non-SCA controls were randomly drawn from the general population using

Table 1. Cardiac arrhythmias observed in patients using CBZ.

Source	Sex/Age (years) of patient	Cardiac arrhythmia reported	CBZ dose (daily) or serum/plasma level
Beermann et al. (1975)	F/66	3rd degree AV block	1200 mg
Herzberg (1978)	F/85	sinus bradycardia	1000 mg
Hamilton (1978)	F/77	sinus bradycardia	1200 mg
Leslie et al. (1983)	M/50	sinus arrest	overdose (20 g), plasma level 62 mg/L (261 μM)
Boesen et al. (1983)	F/72	3rd degree AV block	400 mg
	F/82	SA block	600 mg
	F/86	SA block	400 mg
Benassi et al. (1987)	F/55	3rd degree AV block	800 mg, plasma level 8.5 μg/mL
	F/59	3rd degree AV block	800 mg, plasma level 4.7 μg/mL
Kasarskis et al. (1992)	F/58	bradycardia, AV block, sinus arrest	peak serum level 79.4 μM
Hojer et al. (1993)	M/34	ventricular fibrillation	peak serum level 218 μM
	M/54	AV block	peak serum level 285 μM
	M/83	3rd degree AV block	peak serum level 220 μM
	F/20	QRS widening	peak serum level 176 μM
Schmidt and Schmitz-Buhl (1995)	not reported	bradycardia/AV block (n = 2), cardiac arrest (n = 2)	overdose (dose not reported)
Koutsampasopoulos et al. (2014)	F/82	3rd degree AV block	1200 mg

AV, atrioventricular; F, female; M, male; SA, sinoatrial.

the PHARMO Database Network,[30] which contains, among other things, complete medication data from the community pharmacists across the Netherlands.

Drug dispensing records for drugs prescription were obtained from computerized databases of pharmacists. Use of CBZ was defined as having a drug-dispensing record within 90 days prior to index-date. We chose a period of 90 days, since, in the Netherlands, prescription length for drugs used for chronic disease is 90 days.

For all cases and controls, we included cardiovascular disease and diabetes mellitus in our analyses because these are known risk factors for SCA. We derived cardiovascular disease and diabetes mellitus by using medication use as proxies as we did previously.[31] Cardiovascular disease was defined by use of β-adrenoceptor blockers, calcium channel blockers, diuretics, renin-angiotensin system inhibitors, diuretics, antithrombotics, nitrates and statins. Diabetes mellitus was defined by use of antidiabetics. Patients were considered users of cardiovascular drugs and antidiabetics if there was any drug-dispensing record within 6 months prior to index-date.

2.2 Cellular Electrophysiological Studies

2.2.1 Cell Preparations

Full details of rabbit ventricular and human atrial cell isolation procedures are provided in the Supplementary Material. The investigation using rabbits conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication 85–23, 1996) and was approved by the institutional animal experiments committee. The human atrial cardiomyocytes were isolated from explanted hearts of male patients with end-stage heart failure caused by ischemic cardiomyopathy. All patients were in New York Heart Association functional class IV and received standard therapy for chronic heart failure (Supplementary Table S1). Informed consent was obtained before heart transplantation, and the protocol complied with institutional guidelines.

2.2.2 Action Potentials

APs were measured at 36 ± 0.2°C in modified Tyrode's solution containing (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.5, HEPES 5.0; pH 7.4 (NaOH). Patch pipettes were filled with solution composed of (in mM): K-gluconate 125, KCl 20, NaCl 5.0, K_2ATP 2.0, HEPES 10; pH 7.2 (KOH). Detailed recording procedures are provided in the Supplementary Material. APs were evoked at stimulation rates of 0.2–4 Hz using square 3-ms current pulses through the patch pipette. To reduce variability in the moment of AP upstroke, stimulus amplitude was chosen such that the AP upstroke originated just before the end of the stimulus, as we described previously.[32] The maximal AP upstroke velocity (dV/dt_{max}) was determined from the first derivative of the AP upstroke from which the approximately constant initial dV/dt in response to the stimulus pulse was subtracted (Figure 1A, inset). In addition, we analyzed resting membrane potential (RMP), AP amplitude (APA), and AP duration at 90% repolarization (APD_{90}), as also shown in Figure 1A. AP parameters from 10 consecutive APs were averaged.

2.2.3 Membrane Current Measurements

The L-type Ca^{2+} current ($I_{Ca,L}$), inward rectifier K^+ current (I_{K1}), delayed rectifier K^+ current (I_K), and transient outward K^+ current (I_{to1}) were all measured at 36 ± 0.2°C with the same solutions as used for the AP measurements. However, I_{to1} was measured in the presence of $CdCl_2$ (0.25 mM) to block I_{Na} and $I_{Ca,L}$, thereby also preventing activation of the outward Ca^{2+}-activated Cl^- current.[33] Suppression of these inward and outward currents allows accurate determination of I_{to1}. The whole-cell sodium current (I_{Na}) in freshly isolated cardiomyocytes is an extremely large and fast activating and inactivating membrane current, which for technical reasons cannot be reliably measured at a close-to-physiological temperature and normal Na^+ gradients over the cell membrane (see Berecki et al. (2010) and primary references cited therein).[34] Therefore, we measured I_{Na} at room temperature with modified bath and pipette solutions (including an identical Na^+ concentration in pipette and bath solution), which allowed specific measurements of Na^+ currents only. Bath solution for I_{Na} measurements contained (in mM): NaCl 7.0, CsCl 133, $CaCl_2$ 1.8, $MgCl_2$ 1.2, glucose 11.0, HEPES 5.0, and nifedipine 0.05; pH 7.4 (CsOH). Patch

pipettes for I_{Na} measurements were filled with (in mM): NaCl 3.0, CsCl 133, $MgCl_2$ 2.0, Na_2ATP 2.0, TEA-Cl 2.0, EGTA 10, HEPES 5.0; pH 7.3 (CsOH). The membrane currents were measured with specific voltage clamp protocols as depicted in the insets to Figures 3–5 and described in detail in the Supplementary Material. Recording procedures and data analysis are also described in detail in the Supplementary Material.

2.2.4 Preparation of Carbamazepine

CBZ obtained from Sigma-Aldrich (St. Louis, MO, US) was freshly dissolved every day in dimethyl sulfoxide (DMSO) as 100 mM stock and diluted in the bath solution to the desired concentration just before use. APs and membrane currents were measured in the presence of the vehicle DMSO and after wash-in of CBZ (1, 10, 30, or 100 μM) in the same cardiomyocytes. In order to obtain steady-state conditions, signals were recorded after a 5 min stimulation period, i.e. under baseline conditions, and 5 min after application of CBZ.

2.3 Statistics

Data are presented as mean ± SEM. The association between CBZ and SCA was estimated by calculating the adjusted odds ratio with 95% confidence interval using conditional logistic regression by adjusting for the use of cardiovascular drugs and antidiabetics. For the patch-clamp study, comparisons were made using One-Way ANOVA, One-Way Repeated Measures (RM) ANOVA, or Two-Way RM ANOVA, followed by pairwise comparison using the Student-Newman-Keuls *post hoc* test. For the epidemiological study, differences in baseline values for binary variables between cases and controls were tested using a chi-square test. Differences in baseline values for continuous variables between cases and controls were tested using an independent *t*-test. $p < 0.05$ defined statistical significance.

3 RESULTS

3.1 Carbamazepine Use and the Risk of Sudden Cardiac Arrest

We first conducted a systematic study to establish whether CBZ use is associated with increased risk of SCA in the general population. We identified 5,473 SCA cases, and matched them to 21,866 non-SCA controls. The mean age of the cases was 68.8 years and 69.9% were male. As expected, the prevalence of cardiovascular drugs and antidiabetics was higher among the cases than controls (Table 2). We observed that the proportion of CBZ users was significantly higher among cases ($n = 24$, 0.44%) than among controls ($n = 41$, 0.19%) (Table 3). After adjusting for cardiovascular drugs and antidiabetics, we found that use of CBZ was associated with increased risk of SCA compared with no use of CBZ, with an adjusted odds ratio of 1.90 (95% confidence interval: 1.12–3.24; Table 3).

Table 2. Characteristics of cases and controls.

	Cases (*n* = 5,473)	**Controls (*n* = 21,866)**
Age, years (mean ± SD)	68.8 ± 14.0	68.8 ± 14.0
Male sex	3,823 (69.9%)	15,263 (69.8%)
Cardiovascular pharmacotherapy[a]		
Beta blockers	1,998 (36.5%)	3,839 (17.6%)
Digoxin	295 (5.4%)	334 (1.5%)
Renin-angiotensin system inhibitors	2,073 (37.9%)	4,802 (22.0%)
Calcium channel blockers	902 (16.5%)	2,016 (9.2%)
Antithrombotics	2,299 (42.0%)	4,853 (22.2%)
Diuretics	1,590 (29.1%)	2,712 (12.4%)
Nitrates	574 (10.5%)	841 (3.9%)
Antiarrhythmic drugs class 1 or 3[b]	114 (2.1%)	183 (0.8%)
Antidiabetics	936 (17.1%)	2,145 (9.8%)

[a] Defined as use within six months before index date.
[b] Defined as use within 90 days before index date.

Table 3. Carbamazepine (CBZ) and risk of out-of-hospital cardiac arrest.

	Cases (*n* = 5,473)	**Controls (*n* = 21,866)**	**Crude odds ratio**	**Adjusted odds ratio**
No use of CBZ	5,438 (99.4%)	21,807 (99.7%)	1.0 (reference)	1.0 (reference)
Use of CBZ	24 (0.44%) [a]	41 (0.19%) [a]	2.34 (1.42–3.89[b])	1.90 (1.12–3.24[b])

[a] Not included are 11 cases (0.20%) and 18 control (0.08%) who used CBZ in combination with other antiepileptic drugs.
[b] 95% confidence interval.

3.2 Effects of Carbamazepine on Action Potentials of Rabbit Ventricular Cardiomyocytes

Next, we characterized the effects of 1, 10, 30, and 100 μM CBZ on APs elicited at 1 Hz in rabbit ventricular cardiomyocytes. Figure 1B shows typical APs under baseline conditions (solid line), in the presence of 100 μM CBZ (dashed line), and upon washout of the drug (gray line). Exposure to 100 μM CBZ resulted in substantial alterations in AP morphology in comparison to baseline conditions, particularly a decrease in dV/dt_{max} and APA (as measures of cardiac depolarization) and a slight decrease of APD_{90} (as a measure of cardiac repolarization). The effects were partially reversible upon washout of the drug. Average data are shown in the top panels of Figure 1C, with the individual (paired) data of the 6 cells tested shown in the bottom panels. These data indicate that dV/dt_{max} and APA were significantly decreased by 12.9 ± 3.3% (294 ± 22 (CBZ) vs. 337 ± 20 V/s (baseline)) and 3.6 ± 1.2% (128 ± 6.5 (CBZ) vs. 133 ± 7.2 mV (baseline)), respectively. The effects of CBZ on dV/dt_{max} and APA were concentration dependent

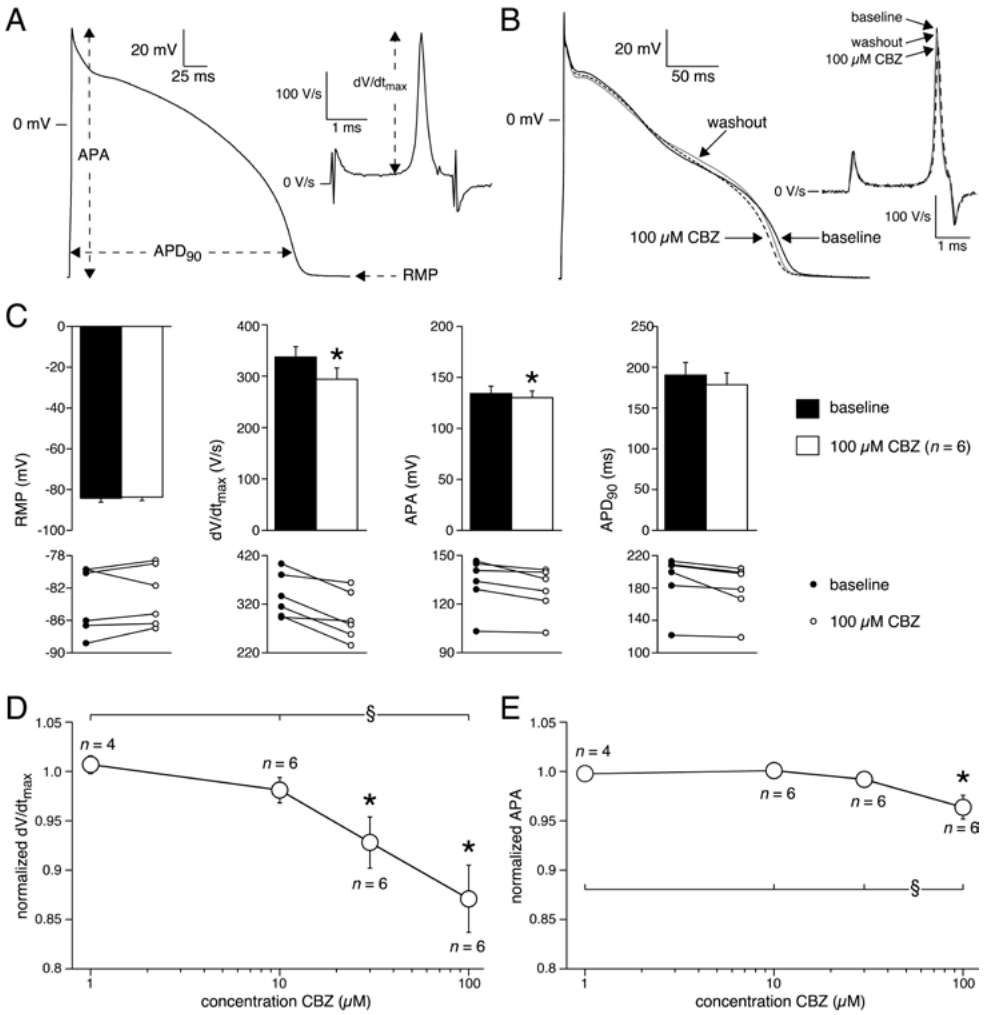

Figure 1. Carbamazepine (CBZ) reduces the action potential (AP) upstroke velocity and AP amplitude of rabbit ventricular cardiomyocytes. **(A)** AP recording illustrating the analyzed AP parameters. Inset: time derivative (dV/dt) of the AP upstroke on an expanded time scale. RMP, resting membrane potential; APA, AP amplitude; APD90, AP duration at 90% of repolarization; dV/dtmax, maximal AP upstroke velocity. **(B)** Superimposed representative APs at 1 Hz under baseline conditions, in presence of 100 μM CBZ, and upon washout of the drug. Inset: time derivatives of the AP upstrokes. **(C)** Average AP characteristics at 1 Hz (top panels) and individual (paired) data points (bottom panels). *$p < 0.05$ CBZ versus baseline (One-Way RM ANOVA). **(D, E)** Average normalized dV/dtmax **(D)** and APA **(E)** at 1 Hz in response to 1, 10, 30, and 100 μM CBZ. Values are normalized to the values measured under baseline conditions. Numbers near symbols indicate the number of cells (*n*) measured at a given concentration. *$p < 0.05$ CBZ versus baseline (One-Way RM ANOVA); § $p < 0.05$ CBZ 100 μM versus lower concentrations (One-Way ANOVA).

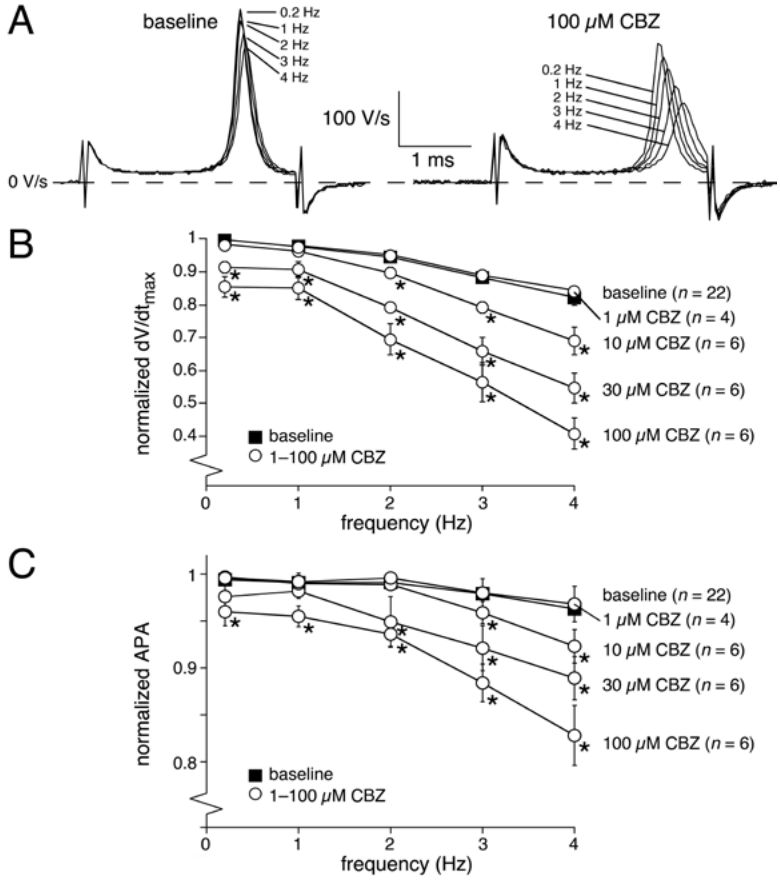

Figure 2. Carbamazepine (CBZ) reduces the AP upstroke velocity and amplitude of rabbit ventricular cardiomyocytes in a frequency dependent manner. (A) Superimposed typical time derivatives (dV/dt) during the AP upstroke phase at stimulus frequencies ranging from 0.2 to 4 Hz under baseline conditions **(left)** and in presence of 100 μM CBZ **(right)**. **(B)** Average dV/dt_{max} under baseline conditions (filled squares) and in response to 1-100 μM CBZ (open circles) at stimulus frequencies ranging from 0.2 to 4 Hz. Values are normalized to the highest dV/dt_{max} measured at 0.2–4 Hz under baseline conditions. *$p < 0.05$ CBZ versus baseline (Two-Way RM ANOVA). See **Supplementary Figure S2** for statistical significance of the frequency dependent effects. **(C)** Average APA under baseline conditions (filled squares) and in response to 1–100 μM CBZ (open circles) at stimulus frequencies ranging from 0.2 to 4 Hz. Values are normalized to the highest APA measured at 0.2–4 Hz under baseline conditions. *$p < 0.05$ CBZ versus baseline (Two-Way RM ANOVA).

(Figures 1D, E). At 100 μM CBZ, RMP was unaffected (–83.0 ± 1.7 (CBZ) vs. –83.5 ± 1.8 mV (baseline)) and the small effect on APD90 (177 ± 14 (CBZ) vs. 189 ± 15 ms (baseline)) did not reach the level of statistical significance (Figure 1C). Similarly, no statistically significant effects on RMP and APD90 were observed at other stimulus frequencies or at lower CBZ concentrations (Supplementary Figure S1).

The upstroke of APs in working cardiomyocytes is mainly due to I_{Na} (see Berecki et al. (2010) and primary references cited therein),[34] which suggests that the CBZ-induced decrease in dV/dt_{max} is due to blockade of I_{Na}. It is well-known that drugs may block I_{Na} in a voltage- and use-dependent manner.[35] The latter means that the amount of block may increase upon higher stimulus frequencies. Figure 2A shows typical AP time derivatives under baseline and 100 μM CBZ conditions at stimulus frequencies ranging from 0.2 to 4 Hz, while Figure 2B summarizes the average effects on dV/dt_{max} at 100 μM CBZ as well as lower concentrations. An increase in stimulus frequency resulted in a significantly lower dV/dt_{max} at every concentration tested (Figure 2B, filled squares; see also Supplementary Figure S2), consistent with a reduced I_{Na} recovery from inactivation at fast pacing rates.[34] In addition, the CBZ-induced decrease in dV/dt_{max} is more pronounced at higher stimulus frequencies (Figure 2B, open circles). For example, 100 μM CBZ decreased dV/dt_{max} by 14.1 ± 3.4% (294 ± 20 (CBZ) vs. 342 ± 18 V/s (baseline)) at 0.2 Hz, but by as much as 41.5 ± 12% (143 ± 21 (CBZ) vs. 264 ± 33 V/s (baseline)) at 4 Hz. Because APA and dV/dt_{max} are both importantly determined by I_{Na},[34, 36] it is not surprising that the APA shows a largely similar concentration and frequency dependency as dV/dt_{max} (Figures 2B, C).

3.3 Effects of Carbamazepine on Membrane Currents of Rabbit Ventricular Cardiomyocytes

We next studied the effects of CBZ on the main membrane currents underlying cardiac APs in rabbit ventricular cardiomyocytes. First, we focused on the main current underlying the AP depolarization, i.e., I_{Na}. Figure 3A shows typical I_{Na} recordings (at –80 to 0 mV) and Figure 3B shows the average current-voltage (I-V) relationships of I_{Na} under baseline conditions and in the presence of 100 μM CBZ. CBZ significantly decreased I_{Na} in the voltage range from –45 to +10 mV, e.g., by 30.3 ± 6.7% at –30 mV (67.8 ± 6.7% (CBZ) vs. 97.3 ± 2.0% (baseline) of the maximal peak amplitude under baseline conditions). Figure 3C shows the dose-dependency of the CBZ effects on I_{Na} and demonstrates that I_{Na} was also significantly reduced by 30 μM CBZ. Figure 3D shows the steady-state activation and inactivation curves for I_{Na} under baseline conditions and in the presence of 100 μM CBZ. While CBZ did not affect the voltage dependency of activation, the voltage dependency of inactivation was significantly shifted to more negative membrane potentials. On average, the negative shift in $V_{1/2}$ was 6.2 ± 1.3 mV (–90.4 ± 1.8 (CBZ) vs. –84.3 ± 1.0 mV (baseline)), while the slope of the inactivation curve was not significantly different between baseline (–5.0 ± 0.9 mV) and CBZ (–5.4 ± 0.7 mV). Figures 3E, F, show the recovery from inactivation of I_{Na}, with in Figure 3E typical I_{Na} recordings (bottom) obtained in response to

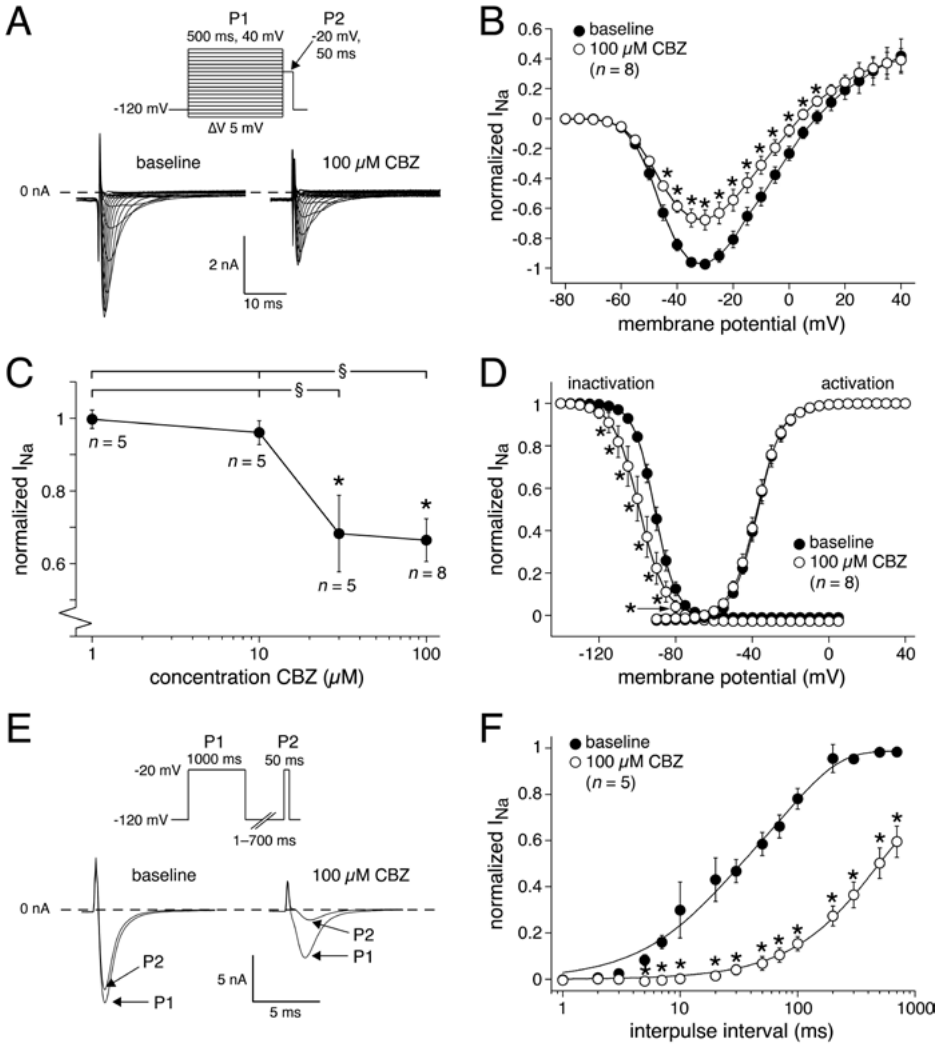

Figure 3. Carbamazepine (CBZ) reduces the sodium current (I_{Na}) of rabbit ventricular cardiomyocytes in a use-dependent manner. **(A)** Typical I_{Na} recordings between -80 and 0 mV under baseline conditions and in presence of 100 μM CBZ. Inset: double-pulse voltage clamp protocol used to measure current-voltage (I–V) relationships **(B)** as well as the voltage dependency of (in)activation **(D)**. Cycle length was 5 s. **(B)** Average I-V relationship of I_{Na} under baseline conditions and in presence of 100 μM CBZ. I_{Na} was normalized to the maximal peak amplitude under baseline conditions, but peak current was set to –1 to retain the well-known inward direction of I_{Na}. *$p < 0.05$ CBZ versus baseline (Two-Way RM ANOVA). **(C)** Concentration dependency of the CBZ effects on I_{Na} amplitude at –35 mV *$p < 0.05$ CBZ versus baseline (One-Way RM ANOVA); § $p < 0.05$ higher versus lower CBZ concentrations (One-Way ANOVA). **(D)** Voltage dependency of (in)activation. Solid lines are Boltzmann fits to the average data. *$p < 0.05$ CBZ versus baseline (Two-Way RM ANOVA). **(E, F)** Recovery from I_{Na} inactivation measured with a double-pulse protocol (E, inset). **(E)** Typical I_{Na} recordings under baseline conditions and in presence of 100 μM CBZ with an interpulse interval of 100 ms. **(F)** Average data. Solid lines are double-exponential fits to the average data. *$p < 0.05$ CBZ versus baseline (Two-Way RM ANOVA).

a double-pulse protocol (top) with an interpulse interval of 100 ms, and in Figure 3F the average data with all interpulse intervals tested. CBZ results in a severe delay in the recovery from

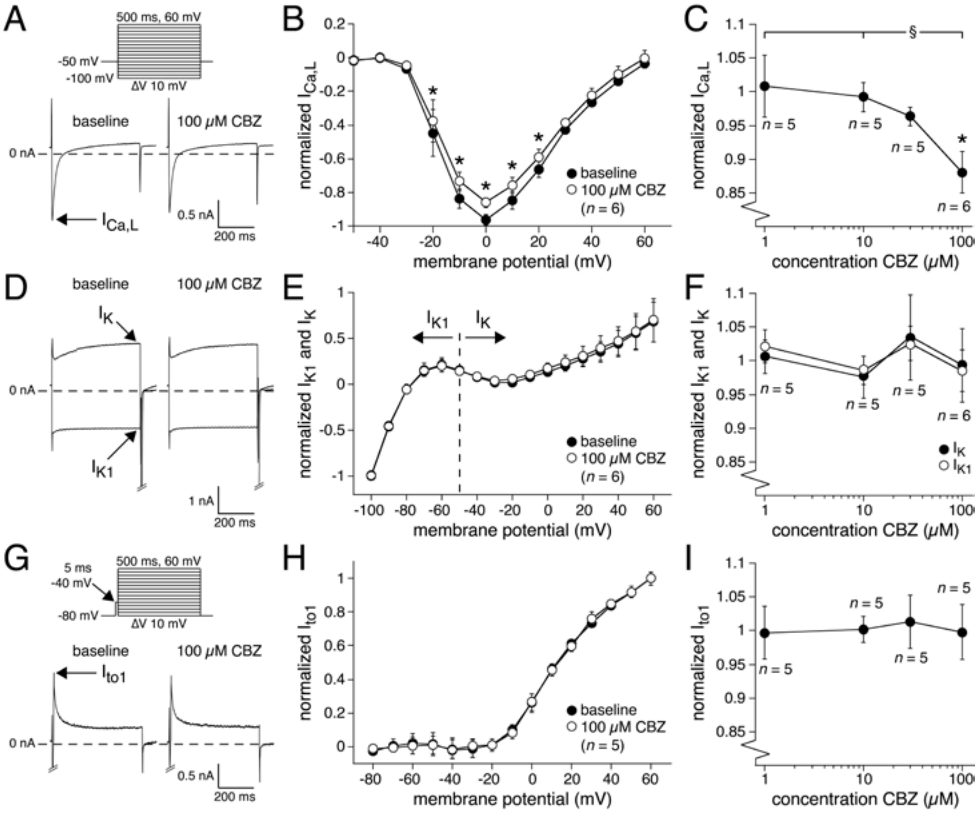

Figure 4. Carbamazepine (CBZ) reduces the L-type Ca^{2+} current of rabbit ventricular cardiomyocytes without affecting K^+ currents. **(A)** Typical recordings of the L-type Ca^{2+} current ($I_{Ca,L}$) at 0 mV under baseline conditions and in presence of 100 μM CBZ. Inset: voltage clamp protocol used. Cycle length was 2 s. **(B)** Average I-V relationship of $I_{Ca,L}$ under baseline conditions and in presence of 100 μM CBZ. $I_{Ca,L}$ was normalized to the maximal peak amplitude under baseline conditions, but peak current was set to −1 to retain the well-known inward direction of $I_{Ca,L}$. $^*p < 0.05$ CBZ versus baseline (Two-Way RM ANOVA). **(C)** Concentration dependency of the CBZ effect on $I_{Ca,L}$ amplitude measured at 0 mV. $^*p < 0.05$ CBZ versus baseline (One-Way RM ANOVA); § $p < 0.05$ CBZ 100 μM versus lower concentrations (One-Way ANOVA). **(D)** Typical recordings of the delayed rectifier K^+ current (I_K; at +60 mV) and inward rectifier K^+ current (I_{K1}; at −100 mV) under baseline conditions and in presence of 100 μM CBZ. Voltage clamp protocol as in panel **(A)**. **(E)** Average I-V relationships of I_K and I_{K1} under baseline conditions and in presence of 100 μM CBZ. The currents were normalized to the current measured at −100 mV (and set to −1) under baseline conditions. **(F)** Concentration dependency of the CBZ effect on I_{K1} and I_K amplitude measured at −100 mV and +60 mV, respectively. **(G)** Typical recordings of the transient outward K^+ current (I_{to1}) at +60 mV under baseline conditions and in presence of 100 μM CBZ. Inset: voltage clamp protocol used. Cycle length was 5 s. **(H)** Average I-V relationships of I_{to1} under baseline conditions and in presence of 100 μM CBZ. $I_{to}1$ was normalized to the current at +60 mV under baseline conditions. **(I)** Concentration dependency of the CBZ effect on I_{to1} amplitude at +60 mV.

inactivation. For example, with an interpulse interval of 100 ms, recovery from inactivation was as large as 78.1 ± 4.5% at baseline, but only 15.4 ± 3.1% in the presence of CBZ.

Second, we studied the main currents underlying the AP repolarization. Although APD_{90} was not significantly affected by CBZ, a potential increase (or decrease) in outward currents can be balanced by a similar increase (or decrease) in inward currents, or vice versa. Figure 4A shows typical recordings (at 0 mV) and Figure 4B shows the average I-V relationships of the inward $I_{Ca,L}$ under baseline conditions and in the presence of 100 μM CBZ. CBZ significantly decreased the $I_{Ca,L}$ density in the voltage range from −20 to +20 mV (Figure 4B). Figure 4C shows that $I_{Ca,L}$ was only significantly reduced at the highest concentration of CBZ tested, i.e., 100 μM. The reduction in peak $I_{Ca,L}$ at 0 mV was 10.3 ± 3.7% (86.0 ± 3.0% (CBZ) vs. 96.2 ± 3.2% (baseline) of the maximal peak amplitude under baseline conditions). Figure 4D shows typical recordings and Figure 4E

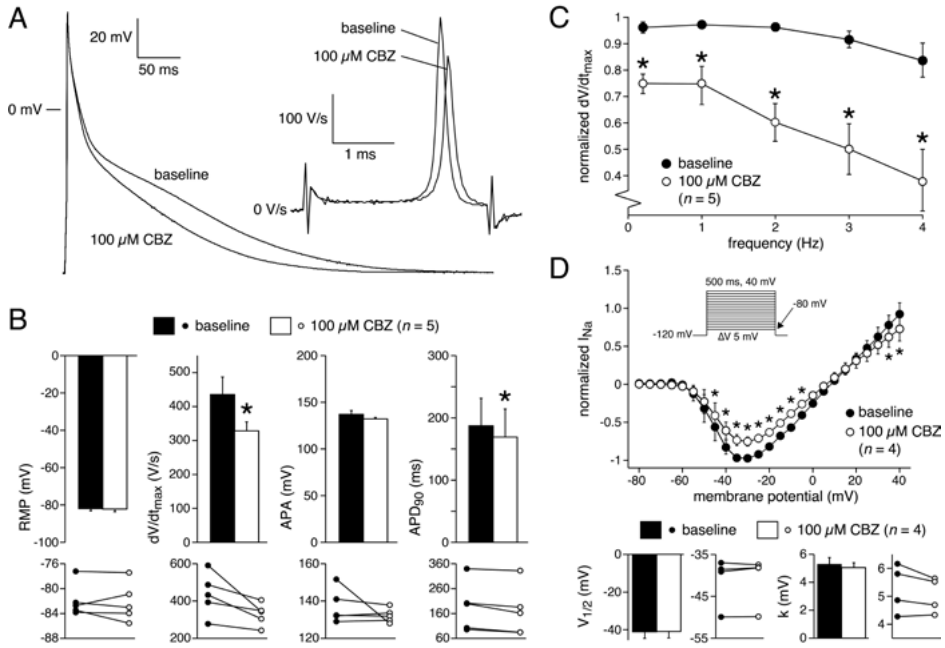

Figure 5. Carbamazepine (CBZ) affects human atrial electrophysiology. **(A)** Superimposed representative human atrial APs at a stimulus frequency of 1 Hz in control conditions and in presence of 100 μM CBZ. Inset: time derivatives during the AP upstroke phase. **(B)** Average AP characteristics at a stimulus frequency of 1 Hz under baseline conditions and in presence of 100 μM CBZ (top panels) and individual (paired) data points (bottom panels). *p < 0.05 CBZ versus baseline (One-Way RM ANOVA). **(C)** Average dV/dtmax in response to 100 μM CBZ at stimulus frequencies ranging from 0.2 to 4 Hz. Values are normalized to the highest dV/dt_{max} measured under baseline conditions. *p < 0.05 CBZ versus baseline (Two-Way RM ANOVA). **(D)** Average I-V relationship of I_{Na} under baseline conditions and in presence of 100 μM CBZ (top panel) and $V_{1/2}$ and k of activation (bottom panels). Inset: voltage clamp protocol used. Cycle length was 5 s. I_{Na} was normalized to the maximal peak amplitude under baseline conditions, but peak current was set to −1 to retain the well-known inward direction of I_{Na}. *p < 0.05 CBZ versus baseline (Two-Way RM ANOVA).

shows the average I-V relationships of the steady-state outward K^+ currents, I_K and I_{K1}, under baseline conditions and in the presence of 100 μM CBZ. Figure 4F shows the concentration dependency of I_K and I_{K1}. Neither I_K nor I_{K1} were significantly affected by CBZ. Figure 4G shows typical recordings and Figure 4H shows the average I-V relationships of I_{to1} under baseline and 100 μM CBZ conditions. Figure 4I shows the concentration dependency of I_{to1}. We observed no significant changes in the amplitude of I_{to1} at any voltage and concentration tested.

3.4 Effects of Carbamazepine on Action Potentials of Human Atrial Cardiomyocytes

Having established the effects of CBZ on AP properties and membrane current of rabbit cardiomyocytes, we measured the effects of 100 μM CBZ on APs and I_{Na} density of freshly isolated human atrial cardiomyocytes to study whether these effects may also occur in the human heart. In patch clamp experiments on single isolated human atrial cardiomyocytes, the amount of quiescent, Ca^{2+}-tolerant cells is typically low and non-depolarized cells are scarce.[33] Here, we selected cardiomyocytes with an RMP of −75 mV or more negative, which generated stable APs after an initial 8–10 min period of continuous pacing at 1 Hz. Figure 5A shows typical APs at 1 Hz under baseline conditions and in the presence of 100 μM CBZ. Average AP parameters are summarized in the top panels of Figure 5B, with the individual (paired) data of the 5 cells tested shown in the bottom panels. Under baseline conditions, the pre-selected human atrial cardiomyocytes had an RMP of −81.9 ± 1.3 mV and a high maximum AP upstroke velocity, and the APs largely overshot the zero potential value. CBZ (100 μM) significantly reduced the AP upstroke velocity and significantly shortened AP duration, without affecting RMP or APA (Figure 5B). These effects are largely comparable to those in rabbit ventricular cardiomyocytes. For example, the AP upstroke velocity decreased significantly by 23.4 ± 6.5% (from 435 ± 58 (baseline) to 328 ± 30 V/s (CBZ)), while the APD_{90} was significantly decreased by 11.8 ± 3.5% (from 187 ± 49 (control) to 169 ± 51 ms (CBZ)). Furthermore, human APs showed a frequency dependency in maximum AP upstroke velocity with a decrease at higher frequencies (Figure 5C, filled circles). The frequency dependency in the presence of CBZ was more pronounced, indicating a similar use-dependent reduction of I_{Na} by CBZ (Figure 5C, open circles) as found in rabbit cardiomyocytes. Figure 5D (top panel), shows the I-V relationships of I_{Na} in human atrial cardiomyocytes under baseline conditions and in presence of 100 μM CBZ. CBZ significantly reduced I_{Na} density, without changes in $V_{1/2}$ and k of activation (Figure 5D, bottom panels).

4 DISCUSSION

The main findings of the present study are: 1) CBZ use is associated with increased SCA risk in the general population; 2) CBZ reduces cardiac AP upstroke velocity and INa in human and rabbit cardiomyocytes; 3) CBZ results in a tendency to (in rabbit) and significant (in human) cardiac AP shortening and reduces $I_{Ca,L}$, while leaving sarcolemmal potassium currents unaltered. All of the observed effects are consistent with each other: reduction in cardiac AP

upstroke velocity is well explained by reduction in I_{Na},[34] and may, in turn, lead to reduction in cardiac excitability and conduction velocity of the excitation wavefront in the heart, as represented by CBZ-induced QRS interval prolongation.[22] It also facilitates reentrant excitation, VF/VT, and SCA, as shown for the use of class IC antiarrhythmic drugs (potent I_{Na} blockers),[37] and in Brugada syndrome (where 20% of patients have an identifiable loss-of-function mutation in *SCN5A*, the gene that encodes the $Na_V1.5$ α-subunit of the cardiac Na^+ channel).[38] Previous case reports (Table 1) have reported findings that are consistent with these electrophysiological effects of CBZ. Accordingly, we found that CBZ use is associated with a 90% increase in the risk of SCA in the general population. These epidemiological findings are consistent with a previous study by Bardai et al. on the association of SCA with epilepsy and with the use of CBZ, which was conducted in a smaller patient set (10 cases used CBZ and 26 controls were included) and with less certain SCA ascertainment (no ECG documentation). In our study, we had no information regarding the epilepsy status. Hence, we could not adjust for epilepsy in the epidemiological analysis. This is an important limitation considering that epilepsy is associated with increased SCA risk.[39] Therefore, our findings from the epidemiological analysis should be interpreted with caution. However, Bardai et al. found that the AEDs with putative cardiac INa blocking properties such as CBZ are similarly associated with an increased SCA risk.[8] This was not only observed among patients with epilepsy, but also among patients who had no epilepsy (but used AEDs for other indications, e.g., neuralgia). Moreover, the observed association between CBZ and SCA remained unchanged after correction for epilepsy.[8] This suggested that the SCA risk associated with CBZ use resulted from the drug effect rather than from suffering epilepsy *per se*.

Of note, we measured the effects of different concentrations of CBZ (1–100 μM) *in vitro*, including concentrations corresponding to plasma levels that provide anticonvulsant effects (20–40 μM).[40] CBZ displays a high distribution volume, entering the bloodstream from tissue reserves,[41] which, together with the fine end-branches of the vasculature of the heart, would make sure that all cardiomyocytes (not only the cells on the surface) are exposed to the compounds in the blood and the extracellular fluid. Thus, the plasma CBZ concentration is a good measure of the concentration of free CBZ "seen" by the cardiomyocytes in the intact heart and in our in vitro experiments. Kennebäck et al. reported a CBZ plasma concentration of 26.1 ± 5.5 μM (mean ± SD) at a dose of 400 mg/day and 35.6 ± 5.9 μM at 800 mg/day in healthy volunteers.[42] Correspondingly, our study showed that the CBZ-induced reduction of upstroke velocity was present at 10 μM at 2 Hz and faster, and at 30 μM at all pacing frequencies, which is thus within the range of therapeutic concentrations. Our observed reduction of upstroke velocity is consistent with findings in guinea-pig ventricular cardiomyocytes where 75 μM CBZ significantly reduced dV/dt_{max} at 1 Hz frequency stimulation by ≈ 13%.[43]

We here compared our used CBZ concentrations to plasma concentrations in healthy volunteers. However, as reviewed by Bertilsson, a poor correlation between the prescribed dose and the actual plasma concentration of CBZ is found in epileptic patients.[40] Furthermore,

CBZ plasma levels may be affected by several factors, among which age, pregnancy, and pharmacokinetic drug interactions, including interactions with both central nervous system and cardiovascular drugs.[40, 44] Consequently, CBZ plasma levels show a considerable inter-individual variability[40, 44]. On the one hand, plasma levels can be so low that therapeutic efficacy is lost, while on the other hand the therapeutic range of 4–10 or 4–12 μg/mL (17–42 or 17–51 μM, respectively) is exceeded in a substantial percentage of patients treated with CBZ,[45-48] which may have contributed to the observed cardiac arrhythmias of Table 1. Supratherapeutic CBZ plasma levels were found in 4.9% of their patients by Shakya et al.,[45], in 8.6% by Al-Balawi et al. ,[46] in 16% by Eroğlu et al.,[48] and in 2.1% by Grześk et al. [47]

The CBZ-induced changes in upstroke velocity support our epidemiological findings, and suggest that CBZ affects I_{Na}.[34] Indeed, we found that ≥30 μM CBZ reduced cardiac I_{Na} and that it affected various gating properties (hyperpolarizing shift in voltage dependency of inactivation and slower recovery from inactivation). Our finding is supported by previous studies on CBZ's effects on cardiac and neuronal I_{Na}.[49-53] For example, Harmer et al. found an IC_{50} of 152 μM for $Na_V1.5$ channels expressed in CHO cells [52], while IC_{50} values for "brain-type" Na^+ channels expressed in HEK293 cells were 2.5 and 1.6 mM for $Na_V1.3$ and $Na_V1.7$ channels in resting state, respectively.[51] In resting state, tetrodotoxin-resistant (TTX-R) $Na_V1.8$ channels had an IC_{50} of 840 μM in dorsal root ganglion cells.[51] CBZ-induced shift in voltage dependency of inactivation and slowed recovery of inactivation were also observed for $Na_V1.3$, $Na_V1.7$ and $Na_V1.8$ channels.[51, 53] This strengthens the notion that I_{Na} block is a plausible contributing mechanism of increased SCA risk associated with CBZ and likely other AEDs with similar cardiac electrophysiological effects. This notion may serve as a basis to adapt clinical procedures for prescription of CBZ with the aim of reducing SCA risk.[23] This may be achieved by identifying individuals who are vulnerable to this risk when prescription of I_{Na} blocking CBZ is considered. This may be based on identification of the clinical conditions that increase SCA risk in the context of I_{Na} block, similar to guidelines regarding the prescription of I_{Na} blocking (class IC) antiarrhythmic drugs in case of ischemic heart disease and heart failure.[54] Also, procedures to screen for genetic vulnerability (pharmacogenetics) may be developed.[39] Finally, at set out above, CBZ levels are affected by several factors and supratherapeutic CBZ levels have been found in a substantial percentage of CBZ users. Therefore, CBZ concentrations need to be closely evaluated.[41, 44]

While I_{Na} block is a plausible mechanism underlying the higher SCA risk observed during CBZ use, there is less compelling evidence to support the notion that increased SCA risk results from changes in AP repolarization. We found mild effects of CBZ on AP repolarization as indicated by the tendency to (in rabbit cardiomyocytes) and significant (in human cardiomyocytes) APD_{90} shortening at 100 μM CBZ, which is above the reported plasma concentrations.[42] An AP shortening was also observed at 75 μM CBZ in guinea-pig ventricular myocytes at 1 Hz stimulation frequency,[43] but QT intervals, ECG measures of the ventricular AP durations,

were not affected by therapeutic doses of CBZ.[55-58] The mild extent of CBZ effects on AP repolarization fits with our voltage clamp experiments. We observed a lack of CBZ effects on the main cardiac repolarizing currents, I_{K1}, I_K and I_{to1}, consistent with previous findings in other tissues and expression systems.[59-61] CBZ (10–50 μM) had no effect on I_K in rat isolated sympathetic neurons[59] and NG108-15 neuronal cells,[60] while it did not affect Kir2.1 currents,[61] with Kir2.1 as the major Kir isoform of I_{K1} channels in cardiac myocytes. Although one study reported that CBZ inhibited the I_{Kr} tail current, the CBZ dosages used in that study (250–500 μM) were much higher than recommended therapeutic concentrations.[62] We found a mild reduction of the depolarizing current $I_{Ca,L}$ at 100 μM. Although it agrees with findings in cultured rat hippocampus neurons[63] and rat sensory spinal ganglion cells,[64] it is unlikely that such a decrease contributes to the SCA increase and relates to CBZ-induced changes in whole heart parameters, because the reduction is rather small and only observed at 100 μM, which is above the therapeutic plasma concentrations.[42] It has been demonstrated that CBZ reduced connexin43 expression in cultured cardiomyocytes,[64] but more studies are required to determine the exact role of cardiac connexins in the altered ECG parameters and arrhythmias by CBZ use, and our observation of increased SCA.

The effects of CBZ on APs and I_{Na} density of freshly isolated human atrial cardiomyocytes were only tested at 100 μM due to the limited availability of Ca^{2+}-tolerant, non-depolarized cells.[65] We used human atrial cardiomyocytes isolated from explanted hearts of patients (with various medications) with end-stage heart failure caused by ischemic cardiomyopathy. Although such cells may be in a diseased state, the main effects of CBZ on those human atrial cardiomyocytes were largely similar to those on ventricular cardiomyocytes of control rabbits, indicating that the effects of CBZ are also present in human conditions. The K^+ currents and $I_{Ca,L}$ were measured with very general voltage clamp protocols without specific solutions and/or blockers. Although such measurements might also involve small contributions of other membrane currents, the CBZ effects were assessed in paired experiments. In addition, our findings match with CBZ findings on membrane currents in non-cardiomyocytes,[51, 53, 59-63] indicating that the CBZ effects on these (net) currents were reliably characterized.

5 CONCLUSION

CBZ reduces cardiac depolarization by reducing I_{Na}, and inducing an associated reduction of the AP upstroke velocity, in cardiomyocytes at therapeutic plasma concentrations. CBZ also affects cardiac repolarization, by reducing $I_{Ca,L}$, and an associated reduction of AP duration, but only at relatively high concentrations. These electrophysiological effects may contribute to the found increased SCA risk upon CBZ use in the general population.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Medical Ethics Committee of Academic Medical Center Amsterdam. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of Amsterdam.

AUTHOR CONTRIBUTIONS

HT conceived and designed the study. TE structured and carried out the epidemiological studies. AV structured and designed the patch-clamp studies. LJ and AV carried out the patch-clamp experiments. RW carried out the statistical analysis of the patch-clamp data. LJ and TE drafted the first version of the manuscript. All authors contributed to manuscript revision and approved the final version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

1 SUPPLEMENTARY METHODS AND MATERIALS

1.1 Isolation of Cardiomocytes

1.1.1 Rabbit Cardiomyocytes

Male New Zealand White rabbits (3.0–3.5 kg) were sedated and anaesthetized with 0.2 mL/kg Hypnorm® (0.32 mg/mL fentanyl-citrate and 10 mg/mL fluanisone; intramuscular; Janssen Pharmaceutics, Leiden, The Netherlands), heparinized (5000 IU heparin, LEO Pharma, Buckinghamshire, UK), and subsequently killed by injection of 1 mL/kg Nembutal® (60 mg/mL sodium pentobarbital; intravenous; Ceva Santé Animale B.V., Naaldwijk, The Netherlands). Hearts were excised, and transported to the laboratory in cold (4°C) Tyrode's solution containing (in mM): NaCl 128, KCl 4.7, $CaCl_2$ 1.5, $MgCl_2$ 0.6, $NaHCO_3$ 27, Na_2HPO_4 0.4, and glucose 11; pH 7.4 by equilibration with 95% O_2 and 5% CO_2. Subsequently, hearts were mounted on a Langendorff perfusion apparatus and left ventricular midmyocardial myocytes were isolated by enzymatic dissociation from the most apical part of the left midmyocardial ventricular free wall as described previously in detail.[1]

1.1.2 Human Cardiomyocytes

Human atrial cardiomyocytes were isolated from explanted hearts of male patients with end-stage heart failure caused by ischemic cardiomyopathy. Directly after explantation, the hearts were transported to the laboratory in oxygenated modified Tyrode's solution containing (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.5, HEPES 5.0; pH 7.4 (NaOH). Parts of both atria were cut into small cubic chunks (≈1 mm^3), and stored in modified Tyrode's solution (20°C) until the cell isolation procedure was started. To this end, atrial chunks were placed in nominally Ca^{2+}-free Tyrode's solution (20°C), i.e., modified Tyrode's solution without the $CaCl_2$, which was refreshed two times. Then, the chunks were incubated for 30–60 min in nominally Ca^{2+}-free Tyrode's solution (37°C) to which liberase III (0.22–0.25 U/mL; Roche Diagnostics, Mannheim, Germany), elastase (0.21–0.24 U/mL; Serva, Heidelberg, Germany), and pronase E (0.92 U/mL; Serva) were added. During the incubation period, the chunks were triturated through a pipette (tip diameter: 2.4 mm) and at regular intervals, the solution was microscopically examined for the presence of dissociated cardiomyocytes. When single cells appeared, dissociation was stopped, and the chunks were transferred into a modified Kraft-Brühe (KB) solution (20°C), which was refreshed three times. KB was composed of (in mM): KCl 85, K_2HPO_4 30, $MgSO_4$ 5.0, glucose 20, pyruvic acid 5.0, creatine 5.0, taurine 30, b-hydroxybutyric acid 5.0, succinic acid 5.0, BSA 1%, Na_2ATP 2.0; pH 6.9 (KOH). Thereafter, single cells were obtained by trituration of the chunks (pipette tip diameter: 2 mm) for ≈2 min. Single cells were stored at room temperature in modified KB solution until use.

1.2 Patch Clamp Experiments

1.2.1 Recording Procedures

Action potentials (APs) and membrane currents were recorded in quiescent cardiomyocytes with smooth surfaces and clear striations with the ruptured-patch whole-cell configuration of the patch clamp technique using an Axopatch 200B amplifier (Molecular Devices, San Jose, CA, USA). Patch pipettes (borosilicate glass) were heat polished and had a resistance of 2–3 MΩ for recording APs, and Ca^{2+} and K^+ currents, while it was 1.4–1.8 MΩ for Na^+ current (I_{Na}) measurements. Voltage control, data acquisition, and analysis were accomplished using custom software.[2] All signals were low-pass filtered (5 kHz) and digitized at 25 (APs), 10 (Ca^{2+} and K^+ currents), or 33 (I_{Na}) kHz. Cell membrane capacitance (C_m) was estimated as described previously,[1] series resistance was compensated by ≥80%, and potentials were corrected for the estimated liquid junction potential.[3]

1.2.2 Membrane Current Analysis

Membrane currents were measured with specific voltage clamp protocols as depicted in the insets to Figures 3–5. For the L-type Ca^{2+} current ($I_{Ca,L}$), the delayed rectifier K^+ current (I_K), and the inward rectifier K^+ current (I_{K1}), we used a holding potential of −50 mV to inactivate I_{Na} and the transient outward K^+ current (I_{to1}), while I_{to1} and I_{Na} were measured from a holding potential of −80 and −120 mV, respectively. I_{Na} was measured with a double-pulse protocol (Figure 3A). During the first depolarizing pulses (P1), I_{Na} activates and the currents analyzed here are used to determine current-voltage (I-V) relationships and the voltage dependency of activation. The second pulse (P2) is used to determine the voltage dependency of inactivation. Recovery from inactivation was measured with a double-pulse protocol with two depolarizing steps (P1 and P2) from −120 to −20 mV and a variable interpulse interval (Figure 3E). Currents measured during P2 were normalized to currents measured during P1. I_{Na} protocols were applied once every 5 s. To determine the (in)activation characteristics of I_{Na}, current-voltage relationships were corrected for differences in driving force and normalized to maximum peak current. Steady-state activation and inactivation curves were fit using the Boltzmann equation $I/I_{max} = A/\{1.0+\exp[(V_{1/2}-V)/k]\}$ to determine the membrane potential for half-maximal (in) activation $V_{1/2}$ and the slope factor k.

$I_{Ca,L}$, I_K, and I_{K1} were measured using 500-ms hyper- and depolarizing pulses (Figure 4A) once every 2 s. I_{to1} was measured using 500-ms depolarizing pulses applied every 5 s. A 5-ms prepulse to −40 mV (Figure 4G) served to activate and inactivate I_{Na}. We defined $I_{Ca,L}$ and I_{Na} as the difference between the peak inward current and the current at the end of a depolarizing voltage clamp step. I_{K1} was defined as the current at the end of hyperpolarizing voltage steps, whereas I_K was defined as the current at the end of depolarizing voltage steps (Figure 4, D and E). I_K in ventricular myocytes may consists of a slow and a rapid component (I_{Ks} and I_{Kr}, respectively).[4] The presence of I_{Ks} in rabbit ventricular myocytes is debated (see Verkerk et al.[5] and primary references cited therein). Previously, we were unable to detect I_{Ks}, while the I_{Kr} blocker

E-4031 abolished tail current after 4-s depolarizing pulses from −50 to +40 mV completely.[5] As we discussed previously,[5] the absence of I_{Ks} in rabbit ventricular cardiomyocytes is likely related to differentially expressed I_{Ks} in rabbit ventricles with a markedly smaller I_{Ks} at the apex than at the base. In addition, I_{Ks} is much smaller in midmyocardial than in subepicardial and subendocardial myocytes. In our cell preparation method, we used midmyocardial myocytes from the apex of the heart. Therefore, I_K in our experiments is attributed to I_{Kr} rather than to I_{Ks}. I_{to1} was defined as the difference between peak transient outward current and the current at the end of the depolarizing voltage step.

1.2.3 Osmolarity

In previous studies,[6, 7] we have used a freezing-point depression-type osmometer (Knauer, Germany) and found that the measured and calculated osmolarity of solutions were indeed almost identical. The pH was set with »2.2 mM KOH, NaOH, or CsOH, which resulted in a calculated osmolarity of: (1) 314.1 mOsm for modified Tyrode's solution; (2) 320.4 mOsm for the pipette solution used in the AP, K^+ current, and Ca^{2+} current measurements; (3) 309.4 mOsm for the bath solution in the Na^+ current measurements; and (4) 307.4 mOsm for the pipette solution in the Na^+ current measurements.

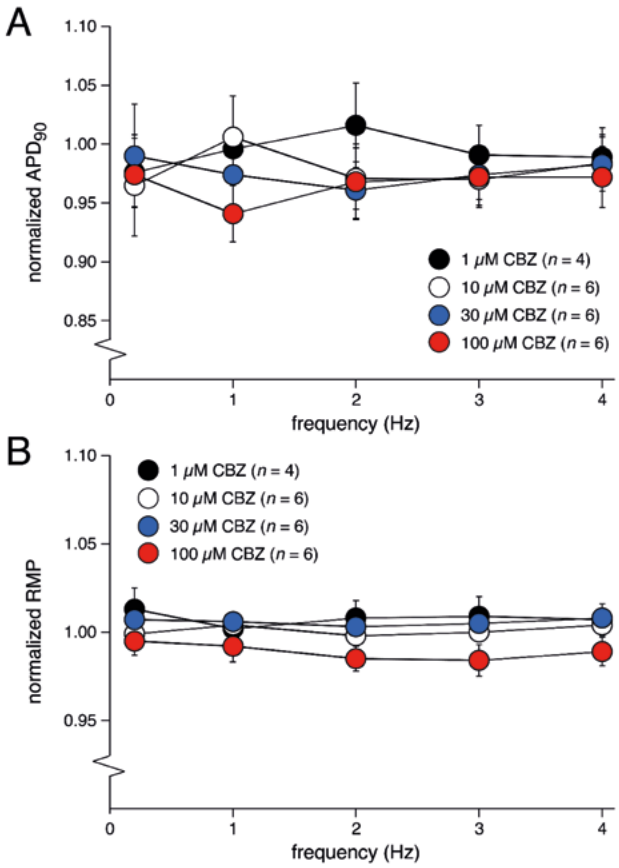
2 SUPPLEMENTARY TABLE

Supplementary Table S1. Data of patients whose hearts were used for cellular electrophysiological studies

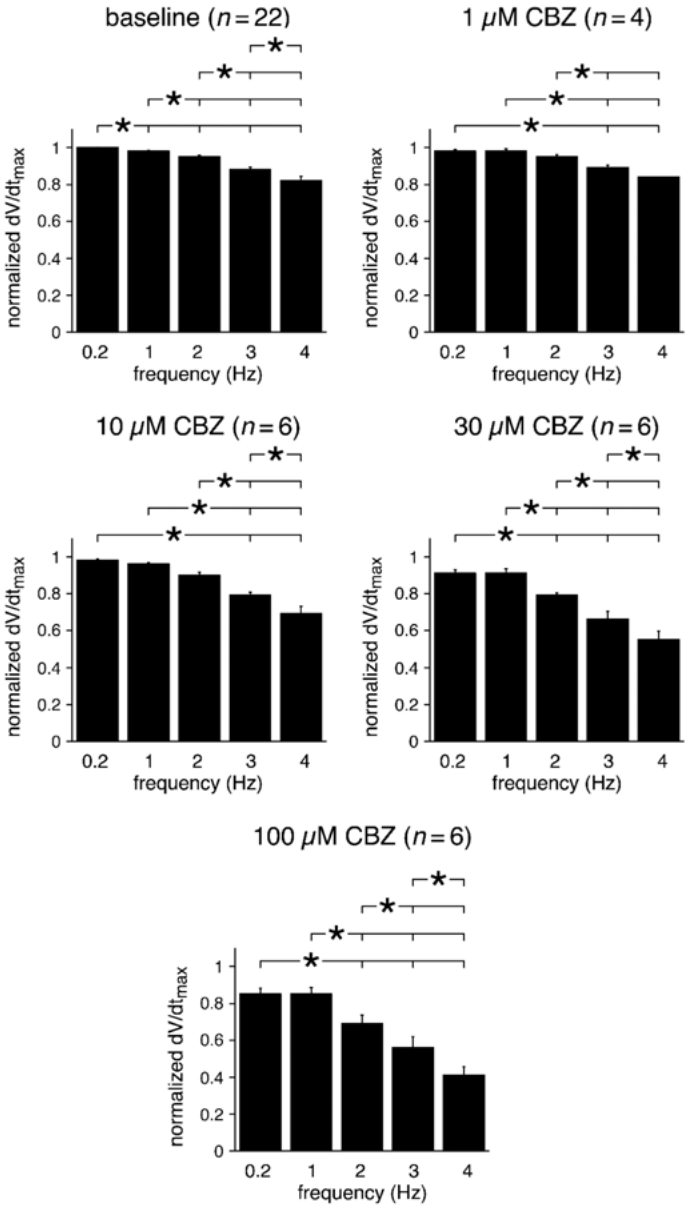
Patient	Age (yr)	Sex	Diagnosis	NYHA class	EF (%)	Clinical data	Previous medications
1	58	M	ICM	IV	19	—	antico, ACE, nit, diu, dig, Ca, dop
2	57	M	ICM	IV	15	VF (ICD)	antico, ACE, nit, diu, dop, b-block
3	61	M	ICM	IV	25	—	antico, ACE, nit, diu

Patient characteristics: NYHA class, New York Heart Association functional class; EF, left ventricular ejection fraction; ICM, ischemic cardiomyopathy; VF, ventricular fibrillation; ICD, implantable cardioverter-defibrillator. Previous medications: antico, anticoagulants; ACE, angiotensin-converting enzyme inhibitors; nit, nitrate; diu, diuretics; dig, digoxin; Ca, Ca-antagonists; dop, dopamine; b-block, b-blockers.

3 SUPPLEMENTARY FIGURES



Supplementary Figure S1. (A) AP duration at 90% repolarization (APD_{90}) and **(B)** resting membrane potential (RMP) of rabbit ventricular cardiomyocytes at stimulus frequencies ranging from 0.2 to 4 Hz in presence of 1 to 100 μM carbamazepine (CBZ). APD_{90} and RMP values are normalized to baseline conditions. $P > 0.05$ at all stimulus frequencies and CBZ concentrations (Two-Way Repeated Measures ANOVA).



Supplementary Figure S2. Frequency dependency of the action potential upstroke velocity (dV/dt_{max}) of rabbit ventricular cardiomyocytes under baseline conditions and in presence of 1 to 100 μM carbamazepine (CBZ). Values are normalized to the highest dV/dt_{max} measured at stimulus frequencies ranging from 0.2 to 4 Hz under baseline conditions. *$P < 0.05$ (Two-Way Repeated Measures ANOVA).

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CHAPTER 3

THE ANTI-EPILEPTIC DRUGS LAMOTRIGINE AND VALPROIC ACID REDUCE THE CARDIAC SODIUM CURRENT

Lixia Jia, Arie O. Verkerk, Hanno L. Tan

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ABSTRACT

Anti-epileptic drugs (AEDs) are associated with increased risk of sudden cardiac death. To establish whether gabapentin, lamotrigine, levetiracetam, pregabalin, and valproic acid reduce the Nav1.5 current, we conducted whole-cell patch-clamp studies to study the effects of the five AEDs on currents of human cardiac Nav1.5 channels stably expressed in HEK293 cells, and on action potential (AP) properties of freshly isolated rabbit ventricular cardiomyocytes. Lamotrigine and valproic acid exhibited inhibitory effects on the Nav1.5 current in a concentration-dependent manner with an IC_{50} of 142 ± 36 and 2022 ± 25 μM for lamotrigine and valproic acid, respectively. In addition, these drugs caused a hyperpolarizing shift of steady-state inactivation and a delay in recovery from inactivation. The changes on the Nav1.5 properties were reflected by a reduction in AP upstroke velocity (43.0 ± 6.8% (lamotrigine) and 23.7 ± 10.6% (valproic acid) at 1 Hz) and AP amplitude; in contrast, AP duration was not changed. Gabapentin, levetiracetam, and pregabalin had no effect on the Nav1.5 current. Lamotrigine and valproic acid reduce the Nav1.5 current density and affect its gating properties, resulting in a decrease of the AP upstroke velocity. Gabapentin, levetiracetam, and pregabalin have no effects on the Nav1.5 current.

1 INTRODUCTION

Anti-epileptic drugs (AEDs) are a mainstay of epilepsy treatment and are also prescribed for behavioral problems and psychiatric disorders.[1] These drugs exert their anti-convulsant actions through various mechanisms, including the blocking of neuronal sodium channels.[2] Of clinical relevance, we and others found that AED use is associated with an increased risk of sudden cardiac death (SCD) due to cardiac arrhythmia.[3, 4] The often-used drug carbamazepine is an example of such a drug.[4] We recently demonstrated that carbamazepine blocks the cardiac sodium channel Nav1.5.[4]

SCN5A encoded Nav1.5 is the most prominent sodium channel in the heart.[5, 6] It is responsible for the rapid upstroke of the cardiac action potential (AP) and regulates impulse propagation in the heart. Nav1.5 block is a plausible mechanism contributing to the elevated SCD risk of cardiac arrhythmia and SCD of carbamazepine[7] because drugs that block the Nav1.5 increase SCD risk.[8, 9] This insight was first gained in the landmark Cardiac Arrhythmia Suppression Trial in which patients randomized to the class 1c cardiac antiarrhythmic drugs (potent Nav1.5 blockers) flecainide or encainide suffered excess mortality rates due to SCD compared to placebo-treated patients.[10] On the other hand, Nav1.5 block by AEDs is plausible given that the Nav1.5 shares great homology with neuronal sodium channel isoforms.[11, 12]

In view of these observations, the aim of our study was to establish whether other AEDs than carbamazepine also block the Nav1.5. Here, we studied the five AEDs which, next to carbamazepine, have the largest number of users in the Netherlands, i.e., gabapentin, lamotrigine, levetiracetam, pregabalin, and valproic acid.[13] We conducted whole-cell patch-clamp studies to evaluate the effects of these drugs on the current densities and gating properties of Nav1.5 channels stably expressed in a HEK293 cell line, and on AP properties of freshly isolated rabbit cardiomyocytes.

2 MATERIALS AND METHODS

2.1. HEK293 Cell Culture

We used a HEK293 cell line with stable human Nav1.5 channel expression.[14] The HEK293 cells were cultured in DMEM with Glutamax (Gibco), supplemented with 10% FBS (Biowest), L-glutamine (Gibco), penicillin-streptomycin (Gibco), and Zeocin (of 200 µg/mL, Invitrogen) in a 5% CO_2 incubator (Shel Lab) at 37 °C. The cells were passaged every 3–4 d at 70% confluency in 25 mL flasks by using 0.25% trypsin (Gibco) treatments of around 2 min. On the day of patch-clamp measurements, cells were trypsined, stored at room temperature, and used within 3 h.

2.2. Rabbit Ventricular Myocyte Preparation

Male New Zealand white rabbits (3.0–3.5 kg) were anesthetized by a combination of ketamine (intramuscular 100 mg) and xylazine (intramuscular 20 mg), heparinized (Heparine LEO 5000 IU), and killed by an injection of pentobarbital (240 mg). Their hearts were excised and transported to the laboratory in cold (4 °C) Tyrode's solution containing (in mM) 128

NaCl, 4.7 KCl, 1.5 $CaCl_2$, 0.6 $MgCl_2$, 27 $NaHCO_3$, 0.4 Na_2HPO_4, and 11 glucose; pH 7.4 by equilibration with 95% O_2 and 5% CO_2. Subsequently, the hearts were mounted on a Langendorff perfusion apparatus and left ventricular midmyocardial myocytes were isolated by enzymatic dissociation from the most apical part of the left midmyocardial ventricular free wall, as described previously.[15] Animal procedures were performed in accordance with governmental and institutional guidelines for animal use in research and were approved by the Animal Experimental Committee of Amsterdam UMC, The Netherlands.

2.3. Patch-Clamp Recording

We applied the whole-cell configuration of the patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices, San Jose, CA, USA). Borosilicate glass patch pipettes (GC100F-10; Harvard Apparatus, UK) had a resistance of 2–3 MΩ after filling with the pipette solution. All signals were low-pass filtered (5 kHz) and digitized at 33 kHz. Series resistance was compensated by ≥80%, and AP potentials were corrected for the calculated liquid junction potential[16] by an offline 15 mV shift in potential toward more negative values. In order to obtain steady-state conditions, signals were recorded after a stable stimulation period, i.e., under baseline conditions, and 5–7 min after the application of AEDs.

2.4. Sodium Current Measurements

The Nav1.5 current was measured in single HEK cells using a pipette solution con-taining (in mM) 10 NaF, 10 CsCl, 110 CsF, 11 EGTA, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 2.0 Na_2ATP, 10 HEPES (pH adjusted to 7.2 with CsOH), and a bath solution containing (in mM) 20 NaCl, 120 CsCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5.5 glucose, and 5.0 HEPES (pH adjusted to 7.4 with CsOH).[17] The Nav1.5 current was measured at room temperature in response to depolarizing voltage steps from a holding potential of −120 mV (cycle length of 5 s). I_{Na} was defined as the difference between peak and steady-state current. The dose-response curves were fitted by the Hill equation: $Y = 1/[(1 + (IC_{50}/X)^n)]$, where Y is the current normalized to baseline condition, IC_{50} is the dose required for 50% current block, and n is the Hill coefficient.

The Nav1.5 (in)activation current was measured with a double-pulse protocol, as detailed in Section 3.2, below. During the first depolarizing pulses (P1), I_{Na} activates, and the currents analyzed here are used to determine current-voltage (I-V) relationships and the voltage dependency of activation. For the latter, the I-V relationships were corrected for driving force and normalized to maximum peak current. The second pulse (P2) is used to determine the voltage dependency of inactivation and currents were normalized to the largest I_{Na}. Voltage dependence of activation and inactivation curves were fitted with the Boltzmann function: $y = 1/\{1 + \exp[(V–V_{1/2})/k]\}$, where $V_{1/2}$ is the midpoint of channel (in)activation, and k is the slope factor of the (in)activation curve. The use-dependent block was determined by application of 30 activating pulses from −120 to −20 mV at a frequency of 4 Hz, as detailed in Section 3.2. The Nav1.5 currents were normalized to the current of the first pulse. The length of recovery from

inactivation was measured with a double-pulse protocol with two depolarizing steps (P1 and P2) from −120 to −20 mV and a variable interpulse interval (see Section 3.2). Currents measured during P2 were normalized to currents measured during P1. Recovery from inactivation was fitted by a double-exponential function: $y = y0 + A_f [1-\exp(-t/\tau_f)] + A_s [1-\exp(-t/\tau_s)]$, where τ_f and τ_s are the fast and slow time constants of recovery from inactivation, respectively, and A_f and A_s are the fractions of fast and slow recovery from inactivation, respectively.

2.5. Action Potential Measurement

APs were measured in single rabbit ventricular cardiomyocytes using the amphotericin-perforated patch-clamp techniques at 36 °C. Cells were superfused with modified Tyrode's solution containing (in mM) 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5.5 glucose, and 5.0 HEPES (pH adjusted to 7.4 with NaOH). Patch pipettes were filled with solution composed of (in mM) 125 K-gluconate, 20 KCl, 5.0 NaCl, 10 HEPES, and 0.44 Amphotericin-B (pH adjusted to 7.2 with KOH). APs were evoked at 1, 2, and 3 Hz using square 3-ms current pulses through the patch pipette. We analyzed resting membrane potential (RMP), AP amplitude (APA), maximal AP upstroke velocity (dV/dt_{max}), and AP duration at 90% repolarization (APD_{90}). AP parameters from 10 consecutive APs were averaged.

2.6. Preparation of Antiepileptic Drugs

All AEDs (purity ≥98%) were purchased from Sigma-Aldrich. Lamotrigine and valproic acid were dissolved in dimethyl sulfoxide (DMSO) to produce a 1 M stock solution. The stock solution was stored at −20 °C and freshly diluted in the bath solution to the desired concentration just before use. The concentration of DMSO in the final solution was less than 0.33% and this does not affect cardiac ion channels.[18, 19] Pregabalin, gabapentin, and levetiracetam were freshly dissolved in the bath solution to the desired concentration just before use. The Nav1.5 current was measured at baseline conditions and after the wash-in of AEDs at concentrations of 1, 10, 30, 100, 300, or 1000 μM; these concentration ranges surrounded the therapeutic concentrations of the AEDs, as indicated by the yellow parts in Figure 1B–F.[20-27]

2.7. Statistical Analysis

Values are presented as mean ± SEM. Curve fitting and statistics was performed using Prim8 GraphPad (GraphPad Software, San Diego, CA, USA). One-Way ANOVA or Two-Way ANOVA was used to assess the statistical significance of the differences among multiple groups. One-way repeated measures (RM) ANOVA was used, followed by pairwise comparison using the Holm-Sidak's multiple comparisons test or One-way RM ANOVA on Ranks (Friedman test), followed by Dunn's multiple comparison test for post hoc analyses when data were not normally distributed. Differences between the two groups were tested using paired Student's t-tests or Two-Way RM ANOVA, followed by pairwise comparison using the Holm-Sidak's multiple comparisons test. Details on normalization are given in Methods or in the figure legends. $p < 0.05$ was considered to be statistically significant.

3 RESULTS

3.1. Inhibition of the Nav1.5 Current by Lamotrigine and Valproic Acid in a Concentration-Dependent Manner

First, we measured the effects of gabapentin, levetiracetam, pregabalin, lamotrigine, and valproic acid on the Nav1.5 current density in HEK293 cells. We applied 100 ms depolarizing pulses from −120 to −40 mV (Figure 1A) to HEK293 cell with stable Nav1.5 expression and tested various drug concentrations including the therapeutic concentrations, which are indicated as yellow parts in Figure 1B–F. We found that lamotrigine and valproic acid reduced the Nav1.5 current density in a concentration-dependent manner (Figure 1E, F). The average IC_{50} of lamotrigine and valproic acid were 142 ± 36 μM (n = 6–7) and 2022 ± 25 μM (n = 5), respectively. The tested concentrations of gabapentin, levetiracetam, and pregabalin had no effect on the Nav1.5 current density (Figure 1B–D).

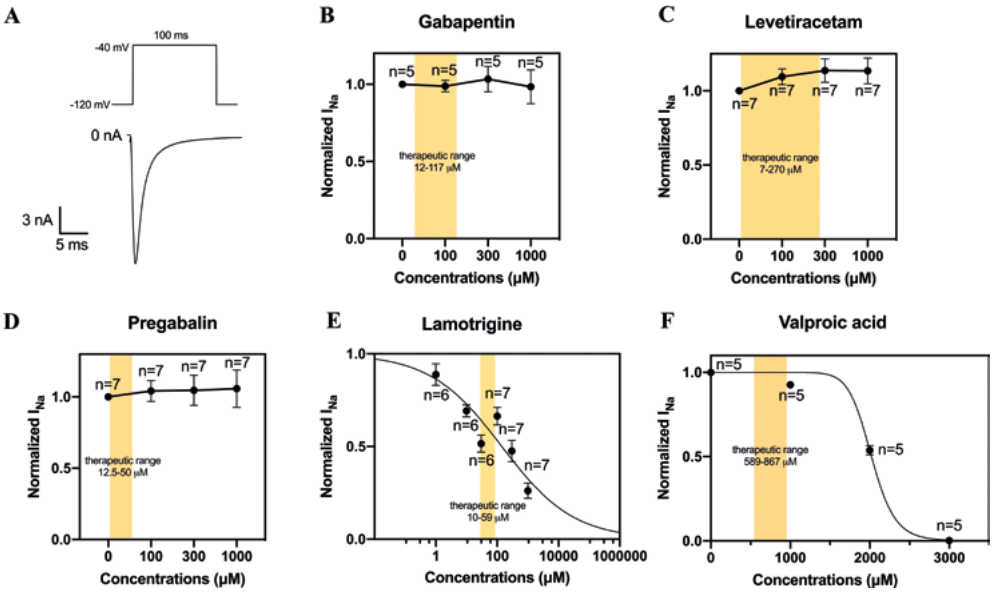

Figure 1. Effects of anti-epileptic drugs on current densities of Nav1.5 channels expressed in HEK293 cells. (**A**) Typical Nav1.5 current elicited by application of 100 ms depolarizing pulses from −120 to −40 mV. (**B–D**) Normalized Nav1.5 current in the absence or presence of 100, 300, or 1000 μM gabapentin (**B**), levetiracetam (**C**), and pregabalin (**D**). (**E, F**) Lamotrigine and valproic acid induced concentration-dependent inhibition of the Nav1.5 current magnitude. Solid lines are Hill fits to the average data. Values are normalized to the values measured under baseline conditions. The yellow parts indicate the therapeutic concentrations of the AEDs. Numbers near symbols indicated the number of cells (n) measured at the given concentrations.

3.2. Effects of Lamotrigine (100 μM) on Gating Properties of Nav1.5 Channels

Second, we tested if the decrease in Nav1.5 in response of 100 μM lamotrigine (close to IC_{50}) is accompanied by changes in gating properties in HEK293 cells. Figure 2A shows typical Nav1.5 currents under baseline conditions and in the presence of 100 μM lamotrigine measured over a wide range of depolarizing voltages (for protocol, see Figure 2A, inset). The average current-voltage (I-V) relationships are shown in Figure 2B. Lamotrigine induced a similar amount of block over the whole voltage range measured and a hyperpolarizing shift in both activation and inactivation (Figure 2C). The $V_{1/2}$ of channel activation occurred at −52.7 ± 1.7 mV in the absence, and −56.7 ± 1.5 mV in the presence, of lamotrigine (n = 9, p < 0.05). The $V_{1/2}$ of channel inactivation were −92.9 ± 1.5 mV and −99.5 ± 2.6 mV, respectively (n = 9, p < 0.05, Table 1). The slope of the inactivation curve was significantly changed after the application of lamotrigine from 6.0 ± 0.4 to 7.7 ± 0.6 mV (n = 9, p < 0.05, Table 1). To study the rate-dependent effects of lamotrigine, we applied a double-pulse protocol with an interpulse interval of 50 ms (Figure 2D) and found that the reduction in the Nav1.5 current density at rising pulse numbers increased more in the presence of lamotrigine (Figure 2E). Consistent with this observation, this was attended by delayed recovery from steady-state inactivation (Figure 2E, Table 1) with τ_f and τ_s significantly changed from 11.2 ± 1.7 to 17.1 ± 3.6 ms, and from 134.8 ± 21.5 to 657.7 ± 125.1 ms, respectively (n = 7, p < 0.05, Table 1).

3.3. Effects of Valproic Acid (2000 μM) on Gating Properties of Nav1.5 Channels

Third, we studied the effects of 2000 μM valproic acid (close to IC_{50}) on the Nav1.5 current in HEK293 cells, similar to that obtained for lamotrigine. Valproic acid reduced the Nav1.5 current density (Figure 3A, B). Figure 3C showed that valproic acid did not induce a statistically significant shift in voltage dependency of activation ($V_{1/2}$ from −50.6 ± 1.5 to −50.5 ± 2.7 mV) (n = 15, p = NS) or slope of activation (Table 1). However, valproic acid induced a significant shift in steady-state inactivation ($V_{1/2}$ from −92.7 ± 1.4 to −99.0 ± 1.9 mV) (n = 15, p < 0.05, Figure 3C, Table 1). And the slope of the inactivation curve was significantly changed after the application of valproic acid from 6.6 ± 0.2 to 5.9 ± 0.3 mV (n = 9, p < 0.05, Table 1). Valproic acid had modest effects on the rate of recovery from in-activation (Figure 3D, E, Table 1), affecting τ_f mildly (from 11.3 ± 2.1 to 17.5 ± 3.6 ms, p < 0.05), but not τ_s (from 165 ± 18.0 to 200.8 ± 34.5 ms, n = 7, p = NS). Accordingly, valproic acid significantly affected the rate of reduction of the Nav1.5 current density at repetitive pacing with an interpulse interval of 50 ms (n = 10, p < 0.05, Figure 3D).

3.4. Effects of Lamotrigine and Valproic acid on Action Potentials Properties

In a final patch clamp experiment, we studied the effects of lamotrigine (100 μM) and valproic acid (3000 μM) on APs elicited in rabbit ventricular cardiomyocytes in order to verify our findings regarding the effects of these AEDs on the Nav1.5 current in HEK293 cells, and to investigate possible drug effects on other ion channels. Figure 4A showed typical APs at 1 Hz

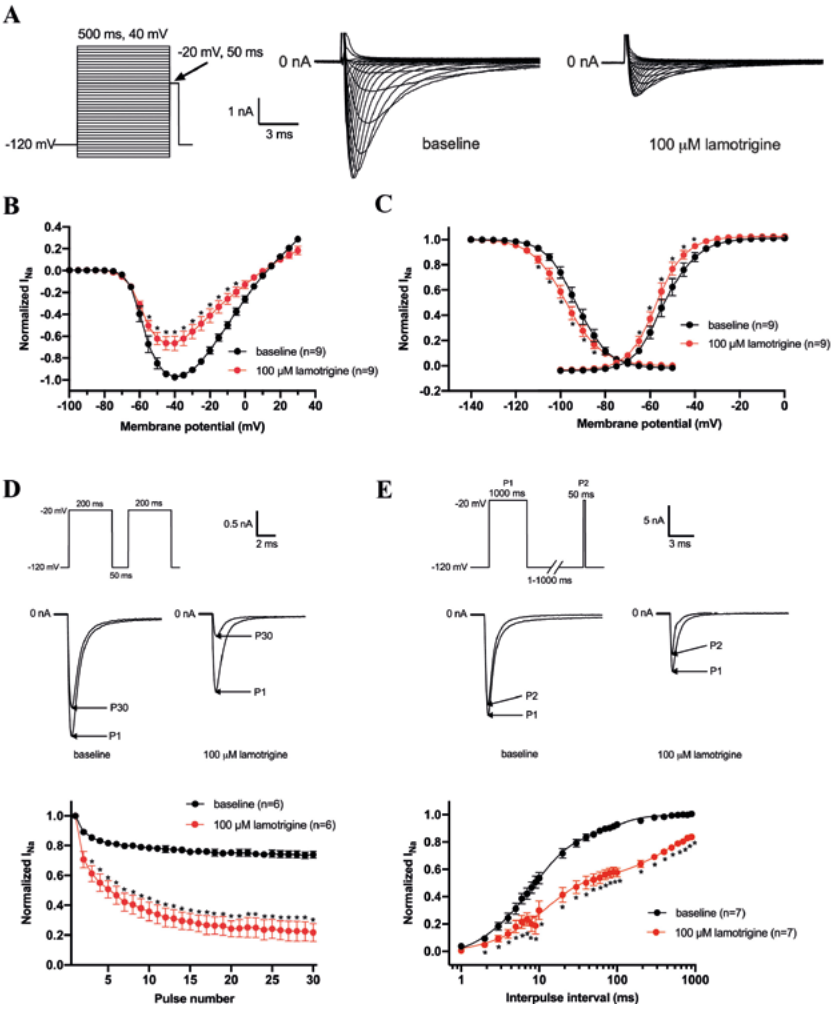

Figure 2. Effects of 100 µM lamotrigine on density and gating kinetics of Nav1.5 channels ex-pressed in HEK293 cells. **(A)** Typical Nav1.5 currents under baseline conditions and in the presence of 100 µM lamotrigine. Inset: voltage clamp protocol used to measure current-voltage (I-V) relationships **(B)** and the voltage dependency of (in)activation **(C)**. Cycle length was 5 s. **(B)** The Nav1.5 current-voltage (I-V) relationships before and after the application of 100 µM lamotrigine. The Nav1.5 current was normalized to the maximal peak amplitude under baseline conditions, but the peak current was set to −1 to retain the well-known inward direction of sodium current. **(C)** Effects of lamotrigine on the voltage dependency of the Nav1.5 current (in)activation. Solid lines are Boltzmann fits to the average data. **(D)** Use dependency under baseline conditions and in the presence of lamotrigine measured during a train of 30 depolarizing pulses with an inter-pulse interval of 50 ms. Inset: voltage clamp protocol used to measure (upper panel) and typical Nav1.5 currents under baseline conditions and in the presence of 100 µM lamotrigine (middle panel). **(E)** Recovery from inactivation of the Nav1.5 current in the absence and presence of 100 µM lamotrigine measured with a double-pulse protocol with variable interpulse intervals. Inset: voltage clamp protocol used to measure (upper panel) and typical Nav1.5 currents under base-line conditions and in the presence of 100 µM lamotrigine with an interpulse interval of 50 ms (middle panel). * $p < 0.05$ lamotrigine versus baseline (Two-Way RM ANOVA).

Table 1. Cardiac sodium current properties in the absence (baseline) and presence of 100 μM lamotrigine and 2000 μM valproic acid.

		Activation		Inactivation		Recovery from Inactivation		
		$V_{1/2}$ (mV)	k (mV)	$V_{1/2}$ (mV)	k (mV)	τ_f (ms)	τ_s (ms)	$A_s/(A_s + A_f)$ (ms)
lamotrigine	baseline	−52.7 ± 1.7	5.7 ± 0.4	−92.9 ± 1.5	6.0 ± 0.4	11.2 ± 1.7	134.8 ± 21.5	0.18 ± 0.02
		(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 7)	(*n* = 7)	(*n* = 7)
	wash-in	−56.7 ± 1.5*	5.1 ± 0.3	−99.5 ± 2.6*	7.7 ± 0.6*	17.1 ± 3.6*	657.7 ± 125.1*	0.44 ± 0.03*
		(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 7)	(*n* = 7)	(*n* = 7)
valproic acid	baseline	−50.6 ± 1.5	5.9 ± 0.3	−92.7 ± 1.4	6.6 ± 0.2	11.3 ± 2.1	165.0 ± 18.0	0.16 ± 0.02
		(*n* = 15)	(*n* = 15)	(*n* = 15)	(*n* = 15)	(*n* = 7)	(*n* = 7)	(*n* = 7)
	wash-in	−50.5 ± 2.7	5.8 ± 0.3	−99.0 ± 1.9*	5.9 ± 0.3*	17.5 ± 3.6*	200.8 ± 34.5	0.2 ± 0.02
		(*n* = 15)	(*n* = 15)	(*n* = 15)	(*n* = 15)	(*n* = 7)	(*n* = 7)	(*n* = 7)

$V_{1/2}$, membrane potential for half-maximal (in)activation; k, slope factor of (in)activation curve; τf and τs, are fast and slow time constant of recovery from inactivation, respectively; and A_f and A_s, fractions of fast and slow recovery from inactivation, respectively. Data are expressed as the mean ± SEM. baseline vs AEDs, * $p < 0.05$ (paired Student's t-tests).

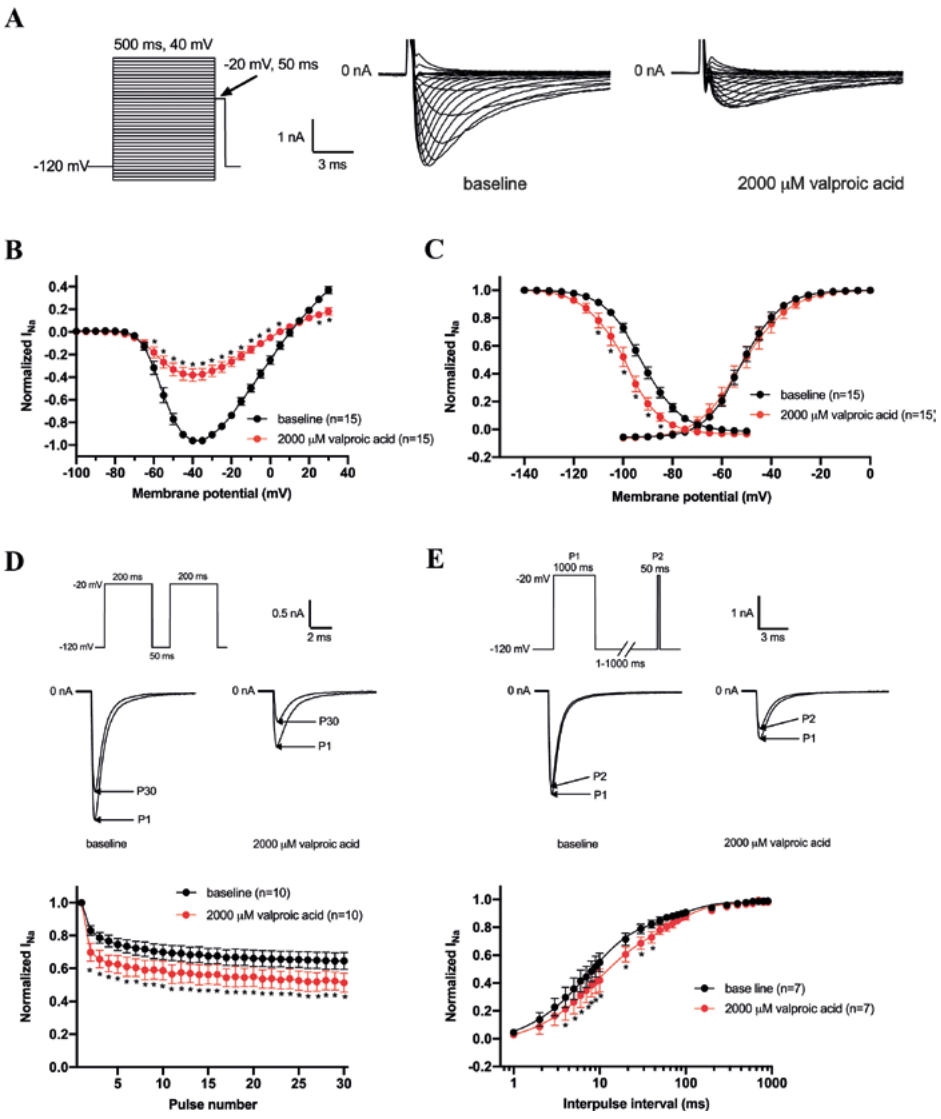

Figure 3. Effects of 2000 µM valproic acid on the density and kinetics of currents from Nav1.5 channels expressed in HEK293 cells. **(A)** Typical Nav1.5 under baseline conditions and in the presence of 2000 µM valproic acid. Inset: used voltage clamp protocol to measure I-V relation-ships **(B)** and the voltage dependency of (in)activation **(C)**. Cycle length was 5 s. **(B)** Average I-V relationships before and after the application of 2000 µM valproic acid. **(C)** Effects of valproic acid on Nav1.5 (in)activation. Solid lines are Boltzmann fits to the average data. **(D)** Use de-pendency under baseline conditions and in the presence of valproic acid. Inset: voltage clamp protocol used to measure (upper panel) and typical Nav1.5 currents under baseline conditions and in the presence of 2000 µM valproic acid (middle panel). **(E)** Recovery from inactivation of the Nav1.5 current in the absence and presence of 2000 µM valproic acid measured with a double-pulse protocol with variable interpulse intervals. Inset: voltage clamp protocol used to measure (upper panel) and typical Nav1.5 currents under baseline conditions and in the presence of 2000 µM valproic acid with an interpulse interval of 50 ms (middle panel). * $p < 0.05$ valproic acid versus baseline (Two-Way RM ANOVA).

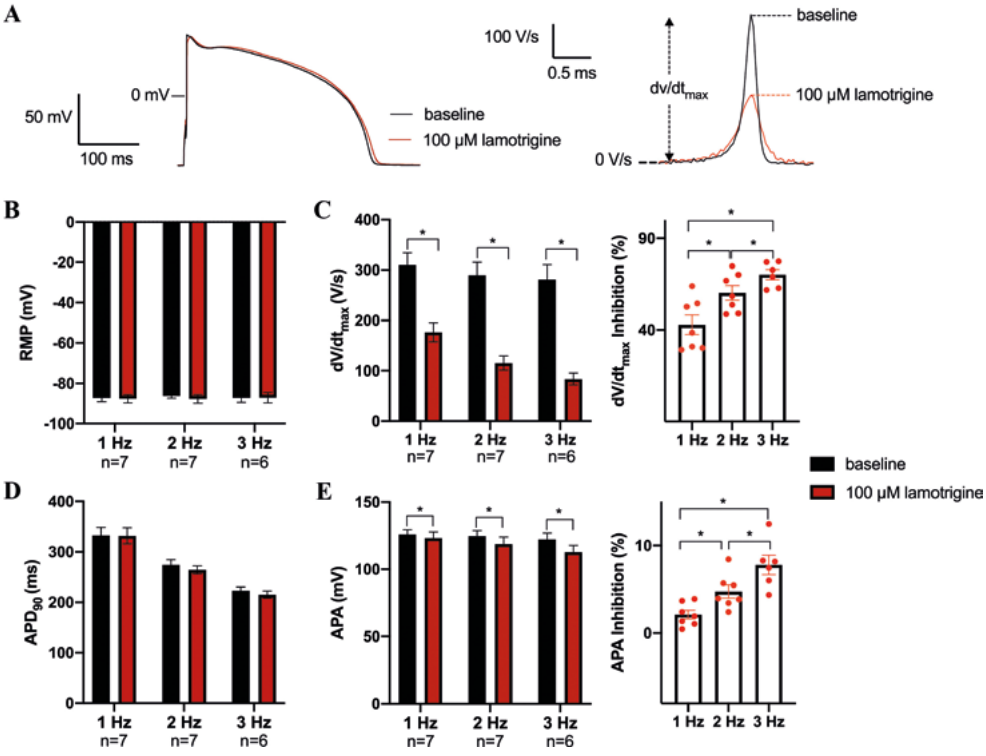

Figure 4. Effects of 100 µM lamotrigine on action potentials (APs) of rabbit ventricular cardio-myocytes. **(A)** Superimposed representative APs at 1 Hz under baseline conditions and in the presence of 100 µM lamotrigine. Inset: time derivatives of the AP upstrokes. The dV/dt_{max} are aligned by the peak. **(B–E)** Average AP characteristics at 1, 2, and 3 Hz of resting membrane potential (RMP, **(B)**), maximal AP upstroke velocity (dV/dt_{max}, **(C, left panel)**) and the inhibition percentage of dV/dt_{max} induced by lamotrigine **(C, right panel)**, AP duration at 90% of repolarization (APD_{90}, **(D)**), AP amplitude (APA, **(E, left panel)**), and the inhibition percentage of APA induced by lamotrigine **(E, right panel)**. Data are mean ± SEM. Numbers below frequencies indicate the number of cells (n) measured at a given frequency. * $p < 0.05$ lamotrigine versus baseline (Two-Way RM ANOVA or One-Way RM ANOVA).

under baseline conditions and in the presence of 100 µM lamotrigine; average AP parameters are summarized in in Figure 4B–D. Lamotrigine caused statistically significant decreases in dV/dt_{max} and APA in a frequency-dependent manner (Figures 4C, E). dV/dt_{max} was significantly decreased at all rates, e.g., by 43.0 ± 6.8% (from 309.7 ± 24.3 to 176.6 ± 19.0 V/s, n = 7, $p < 0.05$) at 1Hz, and by 70.1 ± 8.2% (from 281.2 ± 29.0 to 84.0 ± 11.5 V/s, n = 7, $p < 0.05$) at 3Hz ($n = 7$, $p < 0.05$) (Figure 4C). Similarly, APA decreased by 2.1 ± 0.5% (from 126.0 ± 1.3 to 123.4 ± 1.6 mV, $n = 7$, $p < 0.05$) at 1 Hz, and by 7.8 ± 1.1% (from 122.3 ± 1.9 to 112.8 ± 2.0 mV, $n = 7$, $p < 0.05$) at 3 Hz (Figures 4E). The reduction in dV/dt_{max} was larger at higher stimulation frequencies ($n = 6–7$, $p < 0.05$), consistent with the reduced rate of recovery from inactivation of Nav1.5 (Figure 2D, E). Lamotrigine did not change RMP or APD90. The absence of effects on RMP and APD90 indicates that other ionic currents were virtually unaffected.

Figure 5A shows typical APs under baseline conditions and in the presence of 3000 µM valproic acid at 1 Hz. The average AP parameters at 1 to 3 Hz are summarized in Figure 5B–E. Valproic acid also decreased dV/dt_{max} in a frequency-dependent manner, e.g., by 23.7 ± 10.6% (from 344.0 ± 20.6 to 261.9 ± 32.3 V/s, n = 10, $p < 0.05$) at 1 Hz, and by 37.4 ± 7.8% (253.6 ± 25.1 to 156.7 ± 38.0 V/s), $n = 5$, $p < 0.05$) at 3 Hz (Figure 5C). APA was also reduced, but only statistically significantly at 3 Hz (Figure 5E). There were no statistically significant effects on RMP or APD_{90}, with the exception of a reduction in RMP at 3Hz (Figures 5B, D).

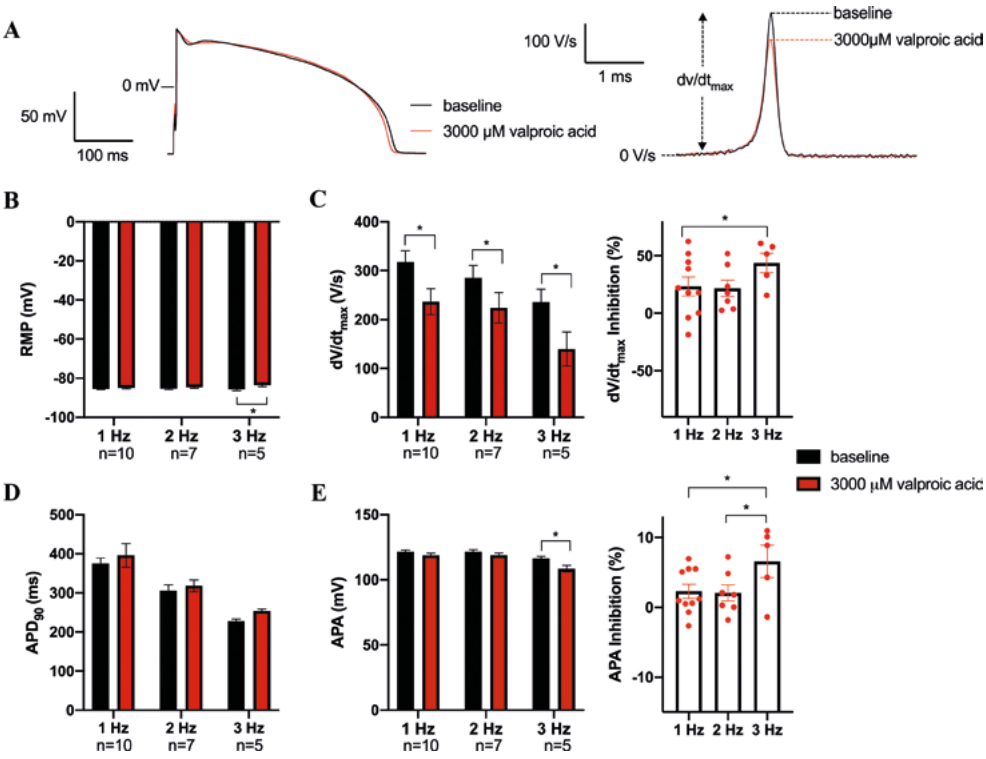

Figure 5. Effects of valproic acid on APs of rabbit ventricular cardiomyocytes. **(A)** Superimposed representative APs at 1 Hz under baseline conditions and in the presence of 3000 µM valproic acid. Inset: time derivatives of the AP upstrokes. The dV/dt_{max} are aligned by the peak. **(B–E)** Average AP characteristics at 1, 2, and 3 Hz of resting membrane potential (RMP, **(B)**), maximal AP upstroke velocity (dV/dt_{max}, **(C, left panel)**), the inhibition percentage of dV/dt_{max} induced by valproic acid **(C, right panel)**, AP duration at 90% of repolarization (APD_{90}, **(D)**), AP amplitude (APA, **(E, left panel)**), and the inhibition percentage of APA induced by valproic acid **(E, right panel)**. Data are mean ± SEM. Numbers below frequencies indicate the number of cells (n) measured at a given frequency. * $p < 0.05$ valproic acid versus baseline (Two-Way RM ANOVA or One-Way RM ANOVA).

4 DISCUSSION

Our main findings were: (1) lamotrigine and valproic acid inhibited the Nav1.5 current in a dose-dependent manner, while gabapentin, levetiracetam, and pregabalin had no effect at the doses tested; (2) lamotrigine and valproic shifted the voltage de-pendency of inactivation and slowed the recovery from inactivation; (3) lamotrigine and valproic acid reduced dV/dt_{max} and APA in rabbit cardiomyocytes with a larger amount of reduction at fast pacing rates; and (4) lamotrigine and valproic acid did not impact other AP properties, except for modest reduction of RMP by valproic acid.

Our observations are largely consistent with reports on the effects of lamotrigine and valproic acid on neuronal sodium channels, reflecting the high homology between cardiac and neuronal sodium channels.[11, 12] We observed a concentration-dependent blockade of the Nav1.5 current by lamotrigine, and similar effects were reported in both cardiac sodium channels expressed in HEK293 cells[28] and neuronal sodium channels expressed in CHO cells.[29] Meanwhile, lamotrigine reduced the density of voltage-gated sodium current in rat cerebellar granule cells and induced a hyperpolarizing shift in the voltage dependency of inactivation,[30] which is consistent with our findings. Our observed lamotrigine-induced delay in recovery from inactivation is also mirrored by similar effects on cardiac sodium channels and neuronal sodium channels.[29] Previous reports on the effects of valproic acid on neuronal sodium channels were also consistent with our findings. For instance, valproic acid reduced sodium current density in the nodal membrane of peripheral nerve fibers of Xenopus laevis by 54% at a dose (2.4 mM) which is very close to our IC_{50} value of the Nav1.5 current block (2.0 mM).[31] Moreover, valproic acid (2 mM) shifted the voltage dependence of inactivation to more hyperpolarized potentials in cortical neurons.[32] Furthermore, valproic acid (1 mM) reduced the sodium current density and slowed the recovery from inactivation in rat hippocampal neurons.[33] While some studies showed that valproic acid had no effect on fast neuronal sodium current, the concentrations used in these studies were mostly lower than therapeutic concentrations.[34, 35]

The antagonizing effects of lamotrigine and valproic acid on the Nav1.5 current, dV/dt_{max}, and APA are here reported for concentrations (100 μM lamotrigine, 3000 μM valproic acid) that exceed the upper limit of the therapeutic range of lamotrigine (59 μM) and valproic acid (867 μM),[27] but only by a factor of 2–3 (while a reduction in sodium current density by lamotrigine already started at concentration within its therapeutic range). Thus, these effects may be clinically relevant because these somewhat elevated concentrations may occur in clinical practice, e.g., at (mild) overdoses. A drug-induced blockade of the human Nav1.5 current could reduce cardiac excitability (as evidenced by a widened QRS complex of the ECG) and increase mortality risk.[36] Accordingly, a review of case reports of lamotrigine overdose found that lamotrigine overdose may be associated with ECG changes (QRS widening) and cardiac arrhythmias (wide complex tachycardia, complete heart block) which are consistent with a reduced Nav1.5 current.[37] Another way in which our findings may have relevance in routine clinical care is that specific subgroups of

patients may have elevated vulnerability to the Nav1.5 blocking effects of lamotrigine or valproic acid.[37] In these individuals, even plasma concentrations within the therapeutic ranges may cause clinically significant effects on cardiac electrophysiology, leading to cardiac arrhythmia and SCD. Enhanced vulnerability may stem from acquired comorbidities and/or from inherited susceptibility. The concept that acquired comorbidities may permit the occurrence of fatal cardiac arrhythmia and SCD upon use of Nav1.5 current blocking drugs was discovered in the Cardiac Arrhythmia Suppression Trial, in which patients randomized to the Nav1.5 current blockers flecainide or encainide suffered excess SCD rates compared to placebo-treated patients.[10] In a meta-analysis, it was discovered that this risk occurred in patients who have increased vulnerability to this adverse drug effect due to comorbidities associated with reduced Nav1.5 function, such as myocardial ischemia/infarction and heart failure.[38] This insight has prompted the recommendation in authoritative clinical guidelines to screen patients for the presence of these comorbidities and withhold these drugs from patients who have them.[39] On the other hand, inherited susceptibility may stem from carrying variants in genes that encode subunits of the Nav1.5 channel, in particular, variants in *SCN5A*, the gene that encodes its α-subunit. Loss-of-function mutations in this gene underlie the Brugada syndrome[40] and cardiac conduction disease,[41] inherited cardiac arrhythmia syndromes associated with elevated SCD risk. Accordingly, mutations in *SCN5A* were found in a series of patients with epilepsy who suffered unexplained and autopsy-negative SCD (sudden unexplained death in epilepsy), and these mutant genes, when heterologously expressed in CHO-K1 cells, produced altered Nav1.5 channel functional properties.[42] Of interest, one of these patients used lamotrigine at the time of SCD.[43] In view of these observations, when prescription of lamotrigine or valproic acid is considered, it could be prudent to first investigate whether the patient has any acquired or inherited condition that would increase the vulnerability to excessive Nav1.5 channel block which could culminate in SCD. This strategy would mirror the strategies in routine cardiology practice to screen patients on these conditions before cardiac drugs that block Nav1.5 channels (e.g., flecainide or other class I antiarrhythmic drugs) are considered,[39] and to withhold these drugs from patients with Brugada syndrome or those who carry *SCN5A* mutations.[44] Screening for inherited vulnerability may be facilitated by the rapidly increasing availability of widespread DNA testing. Before full implementation of DNA testing, inquiring about the presence of familial SCD during simple history taking may already be informative because of the familial nature of SCD.[45] Conversely, the use of lamotrigine and valproic acid may not confer increased risk of cardiac arrhythmias and SCD in patients without enhanced vulnerability.

In any case, when we consider our observations in view of previous pharmacoepidemiologic studies into the associations between AED use and the risk of SCD,[3, 46, 47] we conclude that blocking effects on Nav1.5 channels do not fully account for the increased risk of SCD associated with epilepsy.[48] Our conclusion derives from the fact that our observed effects on Nav1.5 currents were only partly consistent with the findings in these pharmacoepidemiologic studies. For instance, while we report that lamotrigine blocks Nav1.5 currents, this drug was not associated with increased SCD risk in a study of Eroglu et al.[46] While the comparator in

that study was valproic acid, the SCD risk of lamotrigine actually tended to be smaller (but statistical significance was not reached). In the studies of Bardai et al.[46] and Hookana et al.,[47] the numbers were too small to draw conclusions on possible effects of lamotrigine on SCD risk. A recent review also found that there is not sufficient evidence to support or refute the notion that lamotrigine is associated with increased SCD risk.[49] For valproic acid, increased SCD risk was reported by Hookana et al., but not by Bardai et al. (a possible effect on SCD risk was not studied by Eroglu et al., who used valproic acid as comparator). Conversely, while we found that gabapentin, levetiracetam, and pregabalin had no effects on Nav1.5 currents, Eroglu et al. found that pregabalin conferred higher SCD risk than valproic acid, while gabapentin and levetiracetam also tended to have higher SCD risk (but it was not statistically significantly). Similarly, Bardai et al. reported higher SCD risk for gabapentin (for levetiracetam, the statistical power was too small to draw meaningful conclusions). Still, Hookana et al. reported no elevated SCD risk for gabapentin and pregabalin. We conclude from these comparisons that other mechanisms beyond the Nav1.5 block also con-tribute to the elevation in SCD risk in epilepsy, as previously reported.[50]

5 CONCLUSIONS

Lamotrigine and valproic acid reduce the Nav1.5 current by reducing its current density and changing its gating properties; these effects are reflected in changes in AP properties. Gabapentin, levetiracetam, and pregabalin have no effects on the Nav1.5 current.

AUTHOR CONTRIBUTIONS

H.L.T. conceived and designed the study. L.J. and A.O.V. structured and designed the patch-clamp studies. L.J. carried out the patch-clamp experiments, statistical analysis of the patch-clamp data, and drafted the first version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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INSTITUTIONAL REVIEW BOARD STATEMENT

Animal procedures were performed in accordance with governmental and institutional guidelines for animal use in research and were approved by the Animal Experimental Committee of Amsterdam UMC, The Netherlands.

INFORMED CONSENT STATEMENT

Not applicable

DATA AVAILABILITY STATEMENT

Data available in a publicly accessible repository.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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CHAPTER 4

THE OPIOID TRAMADOL BLOCKS THE CARDIAC SODIUM CHANNEL NAV1.5 IN HEK293 CELLS

Lixia Jia, Arie O. Verkerk, Hanno L. Tan

In preparation

ABSTRACT

Background

Opioids are associated with increased risk of sudden cardiac death. This may be due to their effects on the cardiac sodium channel (Nav1.5) current.

4

Objective

To establish whether tramadol, fentanyl, or codeine affect Nav1.5 current.

Methods

Using whole-cell patch-clamp methodology, we studied the effects of tramadol, fentanyl, and codeine on currents of human Nav1.5 channels stably expressed in HEK293 cells, and on action potential (AP) properties of freshly isolated rabbit ventricular cardiomyocytes.

Result

In fully-available Nav1.5 channels (holding potential –120 mV), tramadol exhibited inhibitory effects on Nav1.5 current in a concentration-dependent manner with an IC_{50} of 378.5 ± 33.2 mM. In addition, tramadol caused a hyperpolarizing shift of voltage-gated (in)activation, and a delay in recovery from inactivation. These blocking effects occurred at lower concentrations in partially inactivated Nav1.5 channels: during partial fast-inactivation (close-to-physiological holding potential –90 mV), IC_{50} of Nav1.5 block was 4.5 ± 1.1 μM, while it was 16 ± 4.8 μM during partial slow-inactivation. The tramadol-induced changes on Nav1.5 properties were reflected by a reduction in AP upstroke velocity in a frequency-dependent manner. Fentanyl and codeine had no effect on Nav1.5 current, even when tested at lethal concentrations.

Conclusion

Tramadol reduces Nav1.5 currents, in particular, at close-to-physiological membrane potentials. Fentanyl and codeine have no effects on Nav1.5 current.

1 INTRODUCTION

Opioids are increasingly prescribed for the treatment of severe and chronic pain.[1] The incidence of opioid use disorders due to long-term exposure to opioids is increasing along with death rates associated with unintentional opioid overdoses.[2-4] This may be due to cardiovascular effects of these drugs.[2, 5] For instance, we demonstrated that use of opioid - in particular, tramadol, oxycodone, codeine, fentanyl, morphine and methadone (the six most widely prescribed opioids in the Netherlands) [6] - is associated with an increased risk of sudden cardiac death (SCD) in the general population.[7, 8] This may be due to the ability of these drugs to induce life-threatening cardiac arrhythmias.[5] by impacting on the cardiac ion channels whose concerted activity underlies the cardiac action potential (AP).[9]

Two potentially relevant ion channel targets for opioids are the cardiac sodium channel, Nav1.5, (which is important for AP initiation and propagation).[10] and the hERG potassium channel (crucial for sarcolemma repolarization and AP termination).[11] Increased SCD risk of opioids (in particular, oxycodone, codeine, fentanyl, and morphine) is mostly ascribed to hERG block.[12] and is reflected by QT prolongation of the ECG.[13, 14] The possibility that opioids block Nav1.5 channels is less recognized and studied.[7, 15, 16] Yet, Nav1.5 blockade may also increase SCD risk. Of note, drug-induced Nav1.5 current block and increased SCD risk was not only reported for drugs whose therapeutic effect is based on this effect (Vaughan-Williams class I antiarrhythmic drugs),[16] but also for drugs used for noncardiac disease for which Nav1.5 block is an undesired off-target effect. This is increasingly recognized for a growing number of noncardiac drugs.[7] At present, this group contains tricyclic antidepressants[17, 18] and antiepileptic drugs.[19] We hypothesize that this group may also contain opioids and opioid agonists. Indeed, various opioids, including morphine, U-50,488H, oxycodone, methadone, loperamide, and buprenorphine, inhibit Nav1.5 channels and/or cardiac AP upstroke velocities.[20-25] Currently, the effects of tramadol, codeine, and fentanyl - the most often used opioids in the Netherlands - on Nav1.5 current are unknown. However, tramadol and fentanyl block neuronal (Nav1.2, Nav1.7) voltage-gated sodium channels[24, 26] and because the Nav1.5 isoform is highly homologous with neuronal sodium channels,[27] it is likely that these opioids also reduce Nav1.5 current. The aim of the present study was to establish whether tramadol, codeine or fentanyl reduce Nav1.5 current. To this end, we carried out patch-clamp experiments on human embryonic kidney 293 (HEK293) cells stably expressing human Nav1.5 channels, and determined the effects of the drugs on the APs of freshly isolated rabbit left ventricular cardiomyocytes.

2 MATERIALS AND METHODS

2.1 HEK-293 cell culture

We used a HEK293 cell line with stable human Nav1.5 channel expression.[28] The HEK293 cells were cultured in DMEM with Glutamax (Gibco) supplemented with 10% FBS (Biowest), L-glutamine (Gibco), penicillin-streptomycin (Gibco) and Zeocin (of 200 μg/ml, Invitrogen) in a 5% CO_2 incubator (Shel Lab) at 37 °C. The cells were passaged every 3–4 days at 70%

confluency in 25 ml flasks by using 0.25% trypsin (Gibco) treatments of around 2 mins. On the day of the patch-clamp measurements, cells were trypsined, stored at room temperature, and used within 3 hours.

2.2 Rabbit ventricular cardiomyocyte preparation

Male New Zealand White rabbits (3.0–3.5 kg) were anesthetized by a combination of ketamine (intramuscular 100 mg) and xylazine (intramuscular 20 mg), heparinized (Heparine LEO 5000 IU), and killed by an injection of pentobarbital (240 mg). The hearts were excised, and transported to the laboratory in cold (4 °C) Tyrode's solution containing (in mM): NaCl 128, KCl 4.7, $CaCl_2$ 1.5, $MgCl_2$ 0.6, $NaHCO_3$ 27, Na_2HPO_4 0.4, and glucose 11; pH 7.4 by equilibration with 95% O_2 and 5% CO_2. Subsequently, the hearts were mounted on a Langendorff perfusion apparatus and left ventricular midmyocardial cardiomyocytes were isolated by enzymatic dissociation from the most apical part of the left midmyocardial ventricular free wall as described previously.[29] Animal procedures were performed in accordance with governmental and institutional guidelines for animal use in research and were approved by the Animal Experimental Committee of Amsterdam UMC, The Netherlands.

2.3 Patch-clamp recording

We applied the whole–cell configuration of the patch–clamp technique using an Axopatch 200B amplifier (Molecular Devices, San Jose, CA, USA). Borosilicate glass patch pipettes (GC100F-10; Harvard Apparatus, UK) were pulled using a custom microelectrode puller, and had a resistance of 2-3 MΩ after filling with the pipette solutions (for compositions, see below). All signals were low-pass filtered (5 kHz) and digitized at 33 kHz. Series resistance was compensated by ≥80%. Custom software (Scope, kindly provided by J.G. Zegers, and MacDAQ, kindly provided by A.C.G. van Ginneken) was used to record and analyze Nav1.5 currents and APs.

2.3.1 Sodium current measurements

Nav1.5 current was measured in single HEK293 cells using the ruptured patch-clamp technique at room temperature. The pipette solution contained (in mM): NaF 10, CsCl 10, CsF 110, EGTA 11, $CaCl_2$ 1.0, $MgCl_2$ 1.0, Na_2ATP 2.0, HEPES 10 (pH adjusted to 7.2 with CsOH). The bath solution contained (in mM): NaCl 20, CsCl 120, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.5, HEPES 5.0 (pH adjusted to 7.4 with CsOH).[28] Nav1.5 current was measured in response to depolarizing voltage steps from a holding potential of –120 mV (cycle length of 5 s). At –120 mV, all Nav1.5 channels are fully available for activation, and we named this the 'fully-available' state of the channel. Nav1.5 current was defined as the difference between peak and steady-state current. The dose-response curves were fitted by the Hill equation: $Y=1/[(1+(IC_{50}/X)^n)]$, where Y is the current normalized to baseline condition, IC_{50} is the dose required for 50% current block, and n is the Hill coefficient. Nav1.5 (in)activation current was measured with a double-pulse protocol (see inset of Figure 2B). During the first depolarizing pulses (P1), Nav1.5 current activates and the currents analyzed here are used to determine current-voltage

(I-V) relationships and the voltage dependency of activation. The second pulse (P2) is used to determine the voltage dependency of inactivation. I-V relationships were corrected for driving force, normalized to maximum peak current, and fitted to a Boltzmann distribution curve. Voltage dependence of activation and inactivation curves was fitted with Boltzmann function: $I/Imax = A/\{1.0+\exp[(V_{1/2}-V)/k]\}$, where $V_{1/2}$ is the midpoint of channel (in)activation, and k is the slope factor of the (in)activation curve. The rate of recovery from inactivation was measured with a double-pulse protocol with two depolarizing steps (P1 and P2) from –120 to –20 mV and a variable interpulse interval (see insets of Figure 2E). Currents measured during P2 were normalized to currents measured during P1. Recovery from inactivation curves were fitted by a double-exponential function: $y=y0+A_f\,[1-\exp(-t/\tau_f)]+A_s[1-\exp(-t/\tau_s)]$, where τ_f and τ_s, are the fast and slow time constants of recovery from inactivation, and A_f and A_s are the fractions of fast and slow recovery from inactivation. Use-dependent block was determined by application of 30 activating pulses with a duration of 200 ms from –120 to –20 mV at a frequency of 4 Hz (see insets of Figure 2F). Nav1.5 currents were normalized to the current of the first pulse. In addition to testing the drug effects on fully-available Nav1.5 channels, we also tested drug effects on Nav1.5 channels that were partially inactivated. Effects on Nav1.5 current in partial fast-inactivated state were tested by applying a 1000 ms pre-pulse at –90 mV, followed by a 50 ms test pulse at –40 mV (see inset of Figure 3A). Effects on Nav1.5 current in partial slow-inactivated state were tested by applying a 1000 ms pre-pulse at –40 mV, followed by a 20 ms pulse at –120 mV allowing recovery from fast inactivation, and finally a test pulse to –40 mV (see inset of Figure 3B).

2.3.2 Action potential measurement

APs were measured in single rabbit ventricular cardiomyocytes at 36 °C using the amphotericin-perforated patch-clamp technique. Cells were superfused with modified Tyrode's solution containing (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.5, HEPES 5.0 (pH adjusted to 7.4 with NaOH). Pipette solution contained (in mM): K-gluconate 125, KCl 20, NaCl 5.0, Amphotericin-B 0.44, HEPES 10 (pH adjusted to 7.2 with KOH). APs were evoked at 1 to 3 Hz using square 3-ms current pulses through the patch pipette, and potentials were corrected for the calculated liquid junction potential.[30] We analyzed resting membrane potential (RMP), AP amplitude (APA), maximal AP upstroke velocity (dV/dt_{max}), and AP duration at 90% repolarization (APD_{90}). AP parameters from 10 consecutive APs were averaged.

2.4 Preparation of opioids

Tramadol, fentanyl, and codeine (purity ≥98%), purchased from Sigma-Aldrich, were freshly dissolved in the bath solution to the desired concentration just before use. Nav1.5 current was measured at baseline conditions (no drug) and after 5-8 min wash-in of these drugs at various concentrations that are close to the lethal concentrations of these drugs.[31-33] Tramadol was measured at 1, 10, 30, 100, 300, or 1000 μM; fentanyl was measured at 1 μM; codeine was measured at 100 μM.

2.5 Statistical analysis

Values are presented as mean ± SEM. Curve fitting and statistics was performed using Prim8 GraphPad (GraphPad Software, LLC, USA). One-Way ANOVA or Two-Way ANOVA was used to assess the statistical significance of the differences among multiple groups. One-way repeated measures (RM) ANOVA followed by pairwise comparison using the Holm-Sidak's multiple comparisons test or One-way RM ANOVA on Ranks (Friedman test) followed by Dunn's multiple comparison test for post hoc analyses was used when data was not normally distributed. Differences between two groups were tested using paired Student's t-tests or Two-Way RMs ANOVA followed by pairwise comparison using the Holm-Sidak's multiple comparisons test. Details on normalization are given in Methods or in the figure legends. $P<0.05$ was considered to be statistically significant.

3 RESULTS

3.1 Effects on Nav1.5 current amplitude in HEK293 cells

We first studied whether high (lethal) concentrations of tramadol, fentanyl, or codeine impact Nav1.5 current amplitude in HEK293 cells by applying single 100 ms depolarizing pulses from –120 to –40 mV (Figure 1, A-C). We found that 1 μM fentanyl and 100 μM codeine had no effect on fully-available Nav1.5 current amplitude (Figure 1, B and C). In contrast, 1000 μM tramadol reduced Nav1.5 current amplitude by 69 ± 3.7 % (n=11) (Figure 1A). We therefore conducted additional studies with tramadol to assess its effects on Nav1.5 biophysical properties in more detail.

3.2 Effects of tramadol on current amplitude and gating properties of Nav1.5 channels in HEK293 cells

Using a similar depolarizing step as used in Figure 1, we studied the concentration-dependency of tramadol-induced block (Figure 2A) of fully-available Nav1.5 current and found that the IC_{50} of current reduction was 378.5 ± 33.2 mM. Subsequently, we tested if 300 μM tramadol, a concentration close to the IC_{50}, caused changes in gating properties of Nav1.5. Figure 2B shows typical Nav1.5 currents under baseline condition and in the presence of 300 μM tramadol measured over a wide range of depolarizing voltages (for protocol, see Figure 2B, inset). Figure 2C shows the average I–V relationships, and indicates that tramadol significantly decreased Nav1.5 current in the voltage range from −60 to 0 mV, e.g., by 56.4 ± 4.5% at −40 mV (n=6, $P<0.05$). Tramadol induced a hyperpolarizing shift in voltage dependency of both activation and inactivation (Figure 2D). The average $V_{1/2}$ of channel activation was at –54.6 ± 1.2 mV in the absence, and –61.3 ± 1.6 mV in the presence of tramadol (n=6, $P<0.05$). The average $V_{1/2}$ of channel inactivation were –96.0 ± 2.2mV (baseline) and –105.2 ± 2.7 mV (tramadol), respectively (n=6, $P<0.05$, Table 1). The slope of the activation curve, k, was significantly changed after the application of tramadol from 5.9 ± 0.4 to 5.2 ± 0.3mV (n=6, $P<0.05$, Table 1). To study the rate-dependent effects of tramadol, we applied double-pulse protocols as shown in Figure 2E-F. We found that tramadol delayed recovery from steady-state inactivation

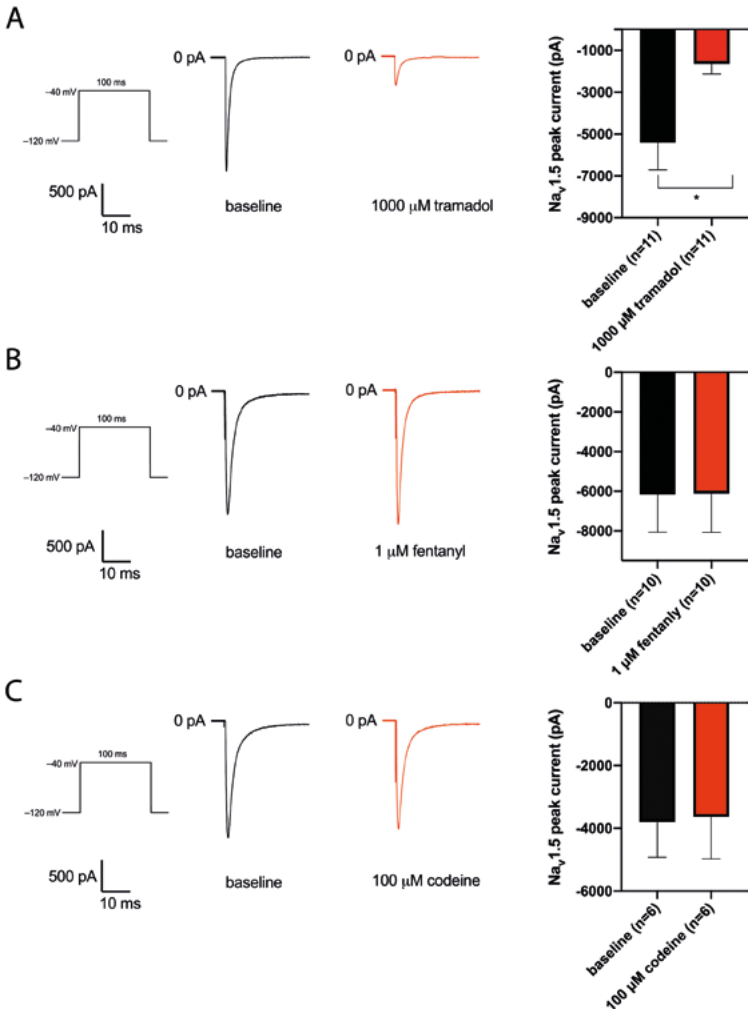

Figure 1. Effects of opioids on current densities of Nav1.5 channels expressed in HEK293 cells. (A-C) Typical Nav1.5 current elicited by application of 100 ms depolarizing pulses from –120 to –40 mV (left panels), and average current amplitudes (right panels) in absence or presence of 1000 µM tramadol (**A**), 1 µM fentanyl (**B**), and 100 µM codeine (**C**). Data are expressed as the mean ± SEM. Numbers indicated the number of cells (n) measured. * $P<0.05$ tramadol versus baseline (paired Student's t-tests).

(Figure 2E, Table 1) with τ_f and τ_s significantly changed from 10.9±2.3 ms to 34.5 ± 8.0 ms, and from 293.6 ± 70.4 ms to 800.1 ± 139.3 ms, respectively (n=5, $P<0.05$, Table 1). Consistent with this observation, the reduction in Nav1.5 current density at rising pulse numbers increased more in the presence of tramadol e.g., by 81.7 ± 3.6 % at the 30th pulse (n=9, $P<0.05$) (Figure 2F).

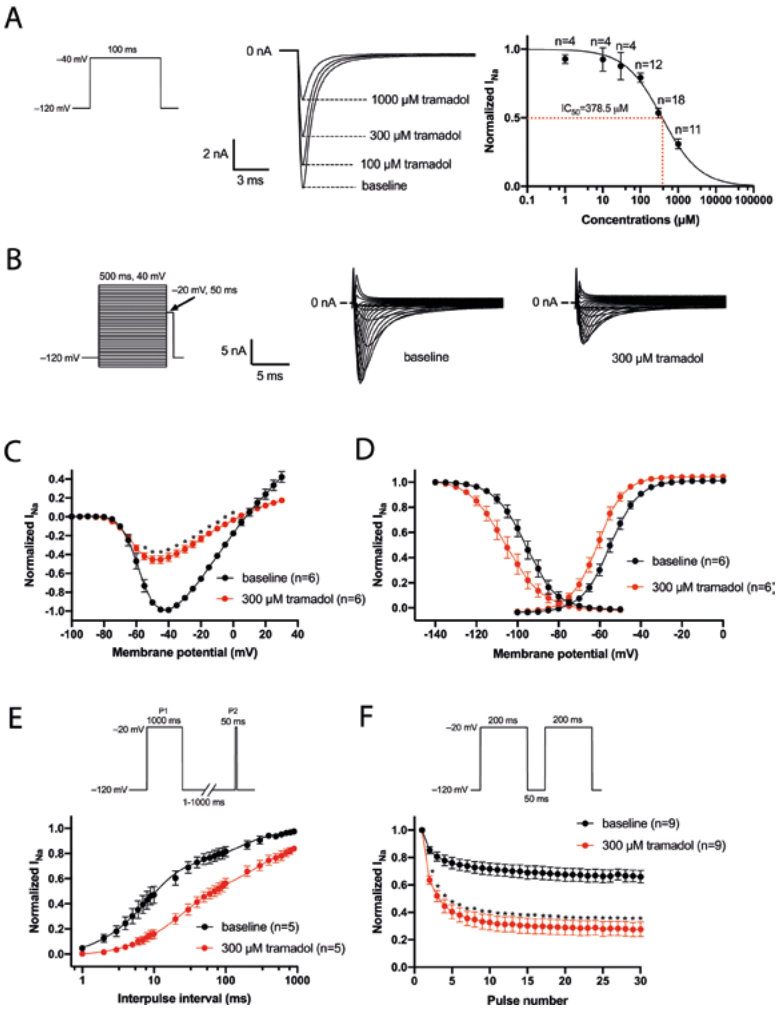

Figure 2. Effects of tramadol on amplitude and gating kinetics of Nav1.5 currents expressed in HEK293 cells. (A) Typical Nav1.5 currents under baseline conditions and in the presence of 100 to 1000 µM tramadol; examples (left panel) and concentration-dependency (right panel) of Nav1.5 current inhibition. The solid line is a Hill fit to the average data. Values are normalized to the values measured under baseline conditions. Numbers near symbols indicated the number of cells (n) measured at the given concentrations. **Inset:** voltage clamp protocol used. **(B)** Typical Nav1.5 currents under baseline conditions and in the presence of 300 µM tramadol. **Inset:** voltage clamp protocol used to measure current-voltage (I-V) relationships and the voltage dependency of (in)activation. Cycle length was 5 s. **(C)** The Nav1.5 I–V relationships before and after the application of 300 µM tramadol. Nav1.5 current was normalized to the maximal peak amplitude under baseline conditions, but peak current was set to –1 to retain the well-known inward direction of the sodium current. **(D)** Effects of tramadol on the voltage dependency of Nav1.5 current (in)activation. Solid lines are Boltzmann fits to the average data. **(E)** Recovery from inactivation of Nav1.5 current in the absence or presence of 300 µM tramadol measured with a double-pulse protocol with variable interpulse intervals (inset). **(F)** Use dependency under baseline conditions and in the presence of tramadol measured during a train of 30 depolarizing pulses with an interpulse interval of 50 ms (inset). * $P < 0.05$ tramadol versus baseline (Two-Way RM ANOVA).

Table 1. Cardiac sodium current properties in absence or presence of 300 μM tramadol

		baseline	tramadol
activation	$V_{1/2}$ (mV)	−54.6±1.2 (n=6)	−61.3±1.6 (n=6) *
	k (mV)	5.9±0.4 (n=6)	5.2±0.3 (n=6) *
inactivation	$V_{1/2}$ (mV)	−96.0±2.2 (n=6)	−105.2±2.7 (n=6) *
	k (mV)	6.6±0.4 (n=6)	7.5±0.6 (n=6)
recovery from inactivation	τ_f (ms)	10.91±2.28 (n=5)	34.52±7.93 (n=5) *
	τ_s (ms)	293.6±70.35 (n=5)	800.1±139.3 (n=5) *
	$A_s/(A_s+A_f)$ (ms)	0.29±0.03 (n=5)	0.47±0.02 (n=5) *

$V_{1/2}$, membrane potential for half-maximal (in)activation; k, slope factor of (in)activation curve. τ_f and τ_s, are fast and slow time constant of recovery from inactivation, respectively; A_f and A_s, fractions of fast and slow recovery from inactivation, respectively. Data are expressed as the mean ± SEM, and n indicates the number of cells. * P<0.05 tramadol versus baseline (paired Student's t-tests).

3.3 Effects of tramadol on density of partially inactivated Nav1.5 current in HEK293 cells

A previous study in neuronal sodium channels showed that tramadol induced more pronounced effects when these channels were in a partially inactivated state than when they were in a fully-available state.[26] To study whether this also occurs in Nav1.5 channels, we studied the concentration dependency of tramadol Nav1.5 current of channels in a partially fast-inactivated or slow-inactivated state in HEK293 cells (Figure 3). The used voltage clamp protocols are shown in the left panels; the middle panels depict typical current traces. Figure 3A showed that tramadol blocked partially fast-inactivated Nav1.5 current in a concentration dependent manner, with an IC_{50} of 4.5 ± 1.1 μM. Figure 3B showed that tramadol blocked partially slow-inactivated Nav1.5 current in a concentration dependent manner, with an IC_{50} of 16 ± 4.8 μM.

3.4 Effects of tramadol on action potentials in rabbit ventricular cardiomyocytes

To verify the functional implication of the tramadol-induced effects on Nav1.5 current, we tested the effects of tramadol (10, 30, 100, and 300 μM) on APs of isolated rabbit left ventricular cardiomyocytes. Figure 4A shows typical APs elicited at 1 Hz under baseline conditions and in the presence of 30 and 300 μM tramadol; average AP parameters after application of 10-300 μM tramadol are summarized in Figure 4, B-E. Tramadol reduced dV/dt_{max} in a concentration dependent manner (Figure 4E), while RMP, APD_{90} and APA were not significantly changed (Figure 4, B-D). Subsequently, we studied the effect of 300 μM tramadol on APs at faster stimulation frequencies. Figure 5A shows typical APs elicited at 1, 2 and 3 Hz under baseline conditions and in the presence of 300 μM tramadol; average AP parameters at 1-3 Hz are summarized in Figure 5, B-E. Tramadol caused statistically significant decreases in APA and dV/dt_{max} in a frequency-dependent manner (Figures 5, D and E). For example, APA decreased by 4.1 ± 1.5% (from 119.2 ± 1.2 to 114.2 ± 1.0 mV, n=6, P<0.05) at 1 Hz, and by 9.0 ± 1.3%

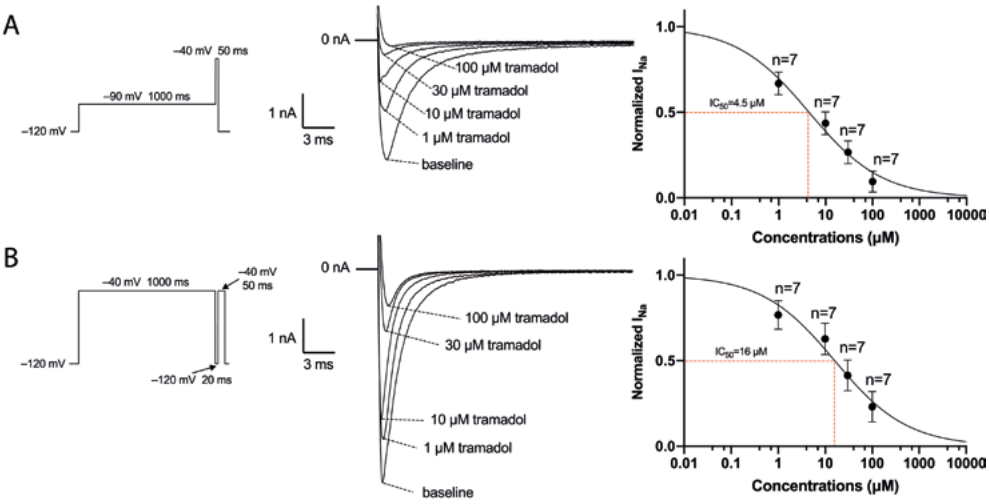

Figure 3. Effects of tramadol on amplitude of inactivated Nav1.5 current in HEK293 cells. (A and B) Block of fast-inactivated **(A)** and slow-inactivated **(B)** Nav1.5 current by tramadol in a concentration-dependent manner. **Inset:** voltage clamp protocol used to measure **(left panel)** and typical Nav1.5 currents under baseline conditions and in the presence of 1 to 100 µM tramadol **(middle panel)**. Cycle length was 5 s. Solid lines are Hill fits to the average data. Values are normalized to the values measured under baseline conditions. Numbers near symbols indicated the number of cells (n) measured at the given concentrations.

(from 119 ± 2.3 to 108.3 ± 2.3 mV, *n*=6, *P*<0.05) at 3 Hz (Figures 5D). Similarly, dV/dt_{max} was significantly decreased at all rates, e.g., by 41.7 ± 9.0% (from 327.7 ± 44.3 to 192.2 ± 37.8 V/s, *n*=6, *P*<0.05) at 1 Hz, and by 58.2 ± 6.7% (from 320.2 ± 51.7 to 136 ± 31.4 V/s, *n*=6, *P*<0.05) at 3 Hz (Figure 5E). Thus, the reductions in APA and dV/dt_{max} were larger at higher stimulation frequencies (*n*=6, *P*<0.05), consistent with the reduced rate of recovery from inactivation of Nav1.5 (Figure 2, E-F). Tramadol did not change RMP or APD_{90} at 1-3 Hz (Figure 5, B and C).

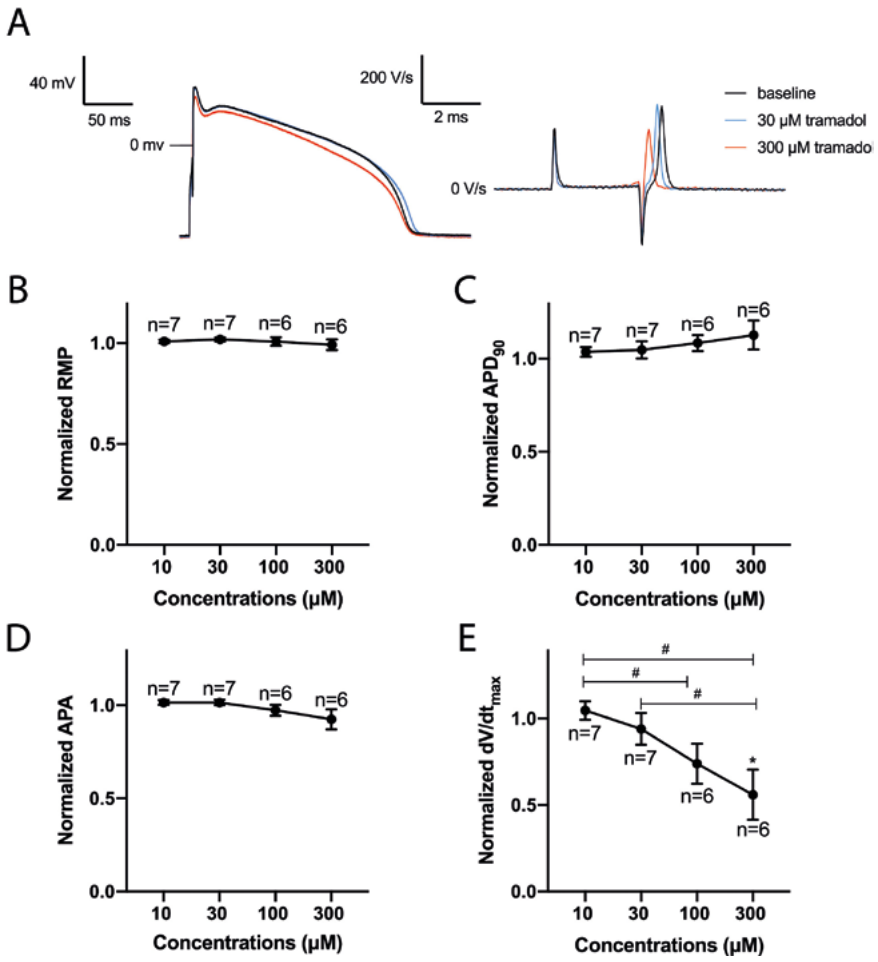

Figure 4. Concentration-dependency of effects of tramadol on action potentials (APs) of single rabbit ventricular cardiomyocytes. **(A)** Superimposed representative APs at 1 Hz under baseline conditions and in the presence of 30 µM and 300 µM tramadol. **Inset:** time derivatives of the AP upstrokes. **(B-E)** Average AP characteristics at 1 Hz of resting membrane potential (RMP, **(B)**), AP duration at 90% of repolarization (APD_{90}, **(C)**), AP amplitude (APA, **(D)**) and maximal AP upstroke velocity (dV/dt_{max}, **(E)**) in absence or presence of 10, 30, 100, or 300 µM tramadol. Data are mean±SEM. Numbers near symbols indicated the number of cells (n) measured at the given concentrations. **P*<0.05 tramadol versus baseline (paired t-test); # *P*<0.05, drug effects observed with different concentrations (One-Way RM ANOVA).

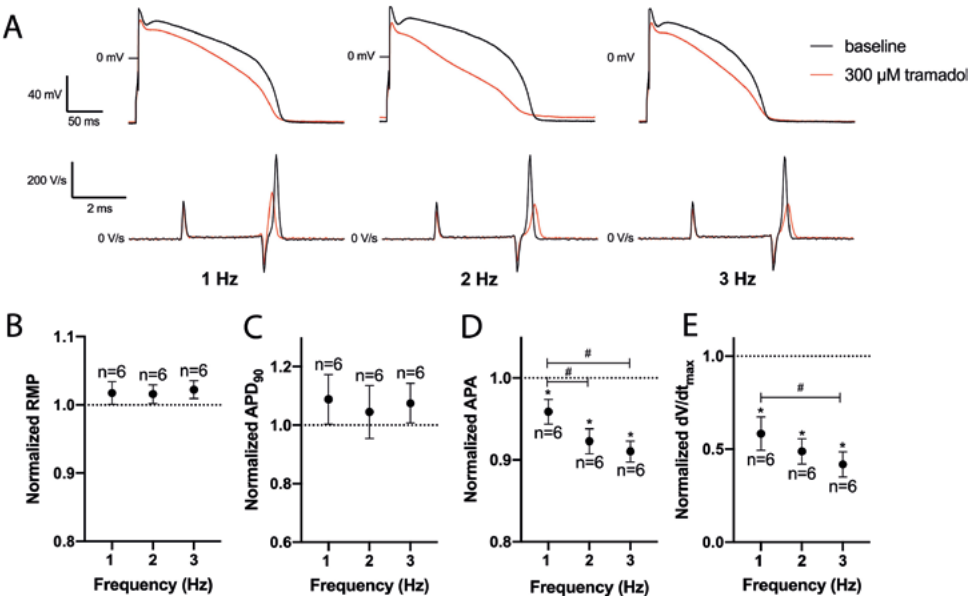

Figure 5. Frequency-dependency of effect of 300 µM tramadol on APs of rabbit ventricular cardiomyocytes. (**A**) Superimposed representative APs at 1, 2 and 3 Hz under baseline conditions and in the presence of 300 µM tramadol. **Inset:** time derivatives of the AP upstrokes. (**B-E**) Average AP characteristics at 1, 2, 3 Hz of RMP (**B**), APD_{90} (**C**), APA (**D**), and dV/dt_{max} (**E**). AP parameters are normalized to the AP parameters measured under baseline conditions in the same cell. Data are mean±SEM. Numbers near symbols indicate the number of cells (n) measured at a given frequency. **P*<0.05 tramadol versus baseline (paired t-test); # *P<0.05*, drug effects observed with different frequencies (One-Way RM ANOVA).

4 DISCUSSION

Our main findings were: (1) tramadol reduced current of Nav1.5 channels in a fully-available state in a dose-dependent manner, while fentanyl and codeine had no effect even when tested at lethal concentrations; (2) tramadol shifted the voltage dependency of (in)activation and slowed the recovery from inactivation of Nav1.5; (3) tramadol caused block of Nav1.5 channels at lower concentrations when these channels were in a partially inactivated state than when they were in a fully-available state; (4) tramadol reduced dV/dt_{max} and APA in rabbit cardiomyocytes with a larger amount of reduction at fast pacing rates. Our results are consistent with previous findings on the effects of tramadol on neuronal sodium channels. Of note, the concentration of tramadol required for 50% inhibition of Nav1.5 currents when measured at a holding potential of –120 mV (IC_{50} 378.5 ± 33.2 µM) was only mildly greater than the concentrations needed to obtain 50% block of TTX-sensitive, neuronal sodium currents in rodent neuroblastoma ND7/23 cells (194 ± 9 µM)[24] and of rat Nav1.2 in HEK293 cells (103 ± 8 µM).[26] Moreover, the tramadol-induced negative shift of voltage-gated inactivation and the use-dependent block of Nav1.5 that we found were also observed in heterologously expressed rat Nav1.2 current.[26] Use of a less negative holding potential increased the blocking potency of tramadol on Nav1.2 current in

HEK293 cells,[26] consistent with our observations in human Nav1.5 current in the present study. Tramadol reduced dV/dt_{max} and APA in rabbit cardiomyocytes in a concentration-dependent and frequency-dependent manner, consistent with our measurement of Nav1.5 current in HEK293 cells.

Our results provide evidence that tramadol has an inhibitory action on Nav1.5 current. This is in line with ECG changes such as QRS widening and Brugada syndrome (BrS) pattern, of patients with tramadol poisoning[34-36] which are importantly due to reduced Nav1.5 channel function.[37, 38] We report that the IC_{50} of tramadol to block fully-available Nav1.5 channels is 378.5 ± 33.2 μM. This is far above the therapeutic range (0.38 to 1.14 μM).[39] When we analyzed tramadol's effects on fully-available Nav1.5 currents, we used a strongly hyperpolarized holding potential of –120 mV. While this method is routinely used for electrophysiological studies, it may hamper extrapolation to physiological conditions, where the RMP of cardiac myocytes is around –80 mV. In fact, at such potentials, many Nav1.5 channels are in an inactivated state. Therefore, the effects of drugs, including tramadol, on partially inactivated Nav1.5 channels are clinically more relevant. When we analyzed tramadol's effects on Nav1.5 channels that were partially in a fast-inactivated state (by utilizing a RMP of –90 mV), we found that the inhibition of Nav1.5 current was strongly enhanced. In fact, the IC_{50} on Nav1.5 channels that were in a partially fast-inactivated was now 4.5 ± 1.1 μM, which is close to therapeutic concentrations (up to 1.14 μM). In the meantime, we also notice that this change is not so obvious in the AP measurements which have a RMP of around –80 mV. Our result showed that the tramadol-induced dV/dt_{max} reduction is concentration dependent, and tramadol-induced reduction of dV/dt_{max} was only present at 300 μM in comparison to the baseline condition. However, compared to 10 μM, tramadol could also reduce the dV/dt_{max} at 100 μM (Figure 4E) in rabbit ventricular myocytes. This may be due to the different experiment conditions, such as measurement temperature, the sodium and fluoride concentrations of the used solutions, or the different cell models.[18, 40-42] Even so, at clinically relevant high concentrations, tramadol-induced overdose toxicity still may be achieved in clinical cases. For instance, the tramadol concentrations in peripheral blood were reported from 1.6 mg/l (6.1μM) to 15.1 mg/l (57.3 μM) in an overview report, which is close to our measured IC_{50} of Nav1.5 channels in a partially fast-inactivated or slow-inactivated state.[43] In another report, tramadol fatal concentrations ranged from 0.03 to 134 mg/l (0.1 to 508.7 μM).[32]

While the effects of tramadol on Nav1.5 channels occurred at concentrations that are somewhat higher than therapeutic concentrations, a relevant consideration is that certain patient groups may have increased sensitivity to the Nav1.5 blocking effects of tramadol.[34] This may stem from acquired comorbidities and/or from inherited susceptibility. A highly prevalent acquired condition is myocardial ischemia and infarction.[44] The concept that these comorbidities may permit or facilitate the occurrence of fatal cardiac arrhythmia and SCD upon the use of Nav1.5 current blocking drugs was discovered in the Cardiac Arrhythmia Suppression Trial. In this

trial, patients randomized to the class 1c antiarrhythmic drugs flecainide or encainide - potent Nav1.5 current blockers - suffered excess SCD rates compared to placebo-treated patients.[16] Excess SCD rates occurred in patients with myocardial ischemia.[45] Inherited susceptibility may stem from carrying variants in genes that encode subunits of Nav1.5 channel, in particular, variants in *SCN5A*, the gene that encodes its a-subunit. Loss-of-function mutations in this gene underlie the BrS[46] and cardiac conduction disease,[47] inherited cardiac arrhythmia syndromes associated with elevated SCD risk. Tramadol was reported to block hERG current in NG108-15 neuronal cells (IC_{50} 25 μM)[48] and in neuronal APs modified by 4-aminopyridine (4-AP) in rat sciatic nerves (measured at 4 mM),[49] and to prolong the duration of the QTc interval of the ECG.[50] Nonetheless, in our study, tramadol had no effect on APD_{90} at tested concentrations in rabbit ventricular cardiomyocytes. This suggests that tramadol may not induce a reduction of cardiac potassium current or that a potential decrease in repolarization current is counteracted by a decrease in L-type calcium current ($I_{Ca,L}$) as was demonstrated by Medei and colleagues.[51]

We found no evidence that fentanyl or codeine blocked fully-available Nav1.5 current, even when tested at fatal concentrations (1 μM and 100 μM, respectively), although fentanyl was shown to block neuronal sodium current with IC_{50} of 141 ± 6 μM in rat Nav1.2 current and 153.2 μM in rat cultured thalamic neurons.[26, 52] Our findings are consistent with the study of Tschirhart and colleagues who also found no effects of fentanyl (10 μM) on the sodium current of neonatal rat ventricular myocytes.[53] Taken together, we found evidence that tramadol blocks Nav1.5 current at clinically relevant concentrations. It is possible that this effect contributes to the increased SCD incidence observed among users of these drugs. Conversely, we found no evidence that fentanyl or codeine block Nav1.5 current at clinically relevant concentrations.

5 CONCLUSION

Tramadol reduces Nav1.5 current by reducing its current amplitude and changing its gating properties; these effects are reflected in changes in AP properties. Fentanyl and codeine have no effects on $Na_v1.5$ current, even when tested at fatal concentrations.

AUTHOR CONTRIBUTIONS

HLT conceived and designed the study. LJ and AOV structured and designed the patch-clamp studies. LJ carried out the patch-clamp experiments and statistical analysis of the patch-clamp data, and drafted the first version of the manuscript. All authors contributed to manuscript revision and approved the final version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 5

SULFONYLUREA ANTIDIABETICS ARE ASSOCIATED WITH LOWER RISK OF OUT-OF-HOSPITAL CARDIAC ARREST: REAL-WORLD DATA FROM A POPULATION-BASED STUDY

Talip E. Eroglu*, Lixia Jia*, Marieke T. Blom,
Arie O. Verkerk, Harsha D. Devalla,
Gerard J.J. Boink, Patrick C. Souverein,
Anthonius de Boer, Hanno L. Tan

* Both authors contributed equally

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ABSTRACT

Aims

Out-of-hospital cardiac arrest (OHCA) mostly results from ventricular tachycardia/ventricular fibrillation (VT/VF), often triggered by acute myocardial infarction (AMI). Sulfonylurea (SU) antidiabetics can block myocardial ATP-regulated K^+ channels (K_{ATP} channels), activated during AMI, thereby modulating action potential duration (APD). We studied whether SU drugs impact on OHCA risk, and whether these effects are related to APD changes.

Methods

We conducted a population-based case–control study in 219 VT/VF-documented OHCA cases with diabetes and 697 non-OHCA controls with diabetes. We studied the association of SU drugs (alone or in combination with metformin) with OHCA risk compared to metformin monotherapy, and of individual SU drugs compared to glimepiride, using multivariable logistic regression analysis. We studied the effects of these drugs on APD during simulated ischaemia using patch-clamp studies in human induced pluripotent stem cell-derived cardiomyocytes.

Results

Compared to metformin, use of SU drugs alone or in combination with metformin was associated with reduced OHCA risk ($OR_{SUdrugs\text{-}alone}$ 0.6 [95% CI 0.4–0.9], $OR_{SUdrugs\ +\ metformin}$ 0.6 [95% CI 0.4–0.9]). We found no differences in OHCA risk between SU drug users who suffered OHCA inside or outside the context of AMI. Reduction of OHCA risk compared to glimepiride was found with gliclazide (OR_{adj} 0.5 [95% CI 0.3–0.9]), but not glibenclamide (OR_{adj} 1.3 [95% CI 0.6–2.7]); for tolbutamide, the association with reduced OHCA risk just failed to reach statistical significance (OR_{adj} 0.6 [95% CI 0.3–1.002]). Glibenclamide attenuated simulated ischaemia-induced APD shortening, while the other SU drugs had no effect.

Conclusion

SU drugs were associated with reduced OHCA risk compared to metformin monotherapy, with gliclazide having a lower risk than glimepiride. The differential effects of SU drugs are not explained by differential effects on APD.

1 INTRODUCTION

Out-of-hospital cardiac arrest (OHCA) is a leading cause of death in industrialized societies. OHCA is predominantly caused by ventricular tachycardia/ventricular fibrillation (VT/VF) that arises from disruptions in cardiac electrophysiology.[1] Diabetes mellitus is an important risk factor for OHCA.[2] Multiple pathophysiologic changes in diabetes may result in VT/VF, in particular, development of ischaemic heart disease.[2] Myocardial ischaemia may lead to VT/VF by inducing various electrophysiological changes. One key mechanism is change in the duration of the action potential (AP) of ventricular cardiomyocytes. AP-shortening, following in large part from opening of myocardial ATP-regulated K^+ channels (K_{ATP} channels) during ischaemia and acute myocardial infarction (AMI),[3] may facilitate re-entrant excitation and VT/VF.[3] Conversely, AP-shortening during ischaemia and AMI may be a cardioprotective mechanism, and lack thereof may result in intracellular Ca^{2+} overload and delayed afterdepolarizations[3] and/or impaired cell-to-cell transmission of the electrical wavefront;[4] these changes may also culminate in VT/VF. Sulfonylurea (SU) drugs, used commonly to achieve glycaemic control in diabetes, exert their therapeutic action by blocking pancreatic K_{ATP} channels, thereby inducing release of insulin.[5] Importantly, SU drugs may also block myocardial K_{ATP} channels,[5] thereby potentially impacting on AP duration of ventricular cardiomyocytes. We therefore hypothesized that SU drugs impact on the risk of OHCA, especially during AMI. To test our hypothesis, we studied whether the use of SU drugs (alone or in combination with metformin) is associated with reduced OHCA risk compared with metformin (both designed for the treatment of type 2 diabetes), in a population-based case–control study based on data from an emergency medical services (EMS) attended OHCA registry, and stratified our analysis according to the immediate cause of OHCA (presence or absence of AMI), expecting that use of SU drugs is more strongly associated with lower OHCA risk in subgroups of OHCA cases who suffered OHCA in the presence of AMI than in the absence of AMI. Moreover, we studied the association between individual SU drugs (glibenclamide, gliclazide, tolbutamide) compared to glimepiride. In addition, we explored the underlying cellular electrophysiological mechanisms by performing patch-clamp studies in human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs).

2 METHODS

2.1 Study design and setting

We conducted a population-based case–control study. Cases were individuals who suffered OHCA from presumed cardiac causes with ECG-documented VT/VF, drawn from the Amsterdam Resuscitation Studies (ARREST,[6]) registry in the study period 2005–2011. We excluded cases without complete drug-dispensing record 1year before index date (OHCA date), those who suffered OHCA from obvious non-cardiac causes (e.g., trauma, drowning), and those who suffered their second OHCA episode. Each case was matched with up to five non-OHCA controls who were alive on the index date (OHCA date) using risk set matching based on age, sex and index date. From this original case–control data set, we included all persons who had used metformin in monotherapy or any SU drug within 90 days before index

date, and excluded all persons who used insulin (proxy for advanced stage of diabetes) or other oral glucose-lowering drugs (e.g., thiazolidinediones), thereby increasing comparability with respect to underlying diabetes severity. By subselecting all individuals with drug-dispensing for metformin or SU drugs 90 days before the index date from the original case–control data set, the original matching was lost. This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Medical Ethics Committee of Academic Medical Center.

2.2 Data sources

Details of the ARREST registry were reported previously.[6] In short, ARREST is an ongoing population-based registry of all EMS-attended OHCAs in one contiguous study region of the Netherlands, representative for the community at large (~2.6 million inhabitants, urban and rural areas, capture rate >90%). The ARREST study centre is notified by all dispatch centres in the study region of every EMS-attended OHCA. ECGs are collected from the manual defibrillator used by EMS personnel and/or the automated external defibrillator used by first responders or citizen-responders. Information from these ECGs with additional information from the dispatch centres and EMS personnel are used to verify the presence of VT/VF. The immediate cause of VT/VF was retrieved from hospital records and was classified as AMI, no AMI (any other cardiac cause) or unknown, as diagnosed by the treating cardiologist.[6] These data were obtained for those individuals who survived to hospital admission. Drug-dispensing records in the year before OHCA was retrieved from the patients' pharmacist using standardized protocols. Controls were derived from the PHARMO Database Network, which contains drug-dispensing records from community pharmacies.[7] We also obtained complete drug-dispensing records in the year before index date from controls. As virtually all individuals in the Netherlands are registered at a single pharmacy, drug-dispensing records are considered complete.

2.3 Exposure of interest

We studied use of the most commonly prescribed SU drugs in the Netherlands (glibenclamide, glimepiride, gliclazide, tolbutamide) by using the Anatomical Therapeutic Chemical classification (ATC) system (see Table S1 in the Supporting Information for the ATC codes). Use of metformin and SU drugs was defined as having a drug-dispensing record within 90 days before the index date, since, in the Netherlands, the average repeat prescription length for drugs used for chronic diseases is 90 days. We classified users of antidiabetics into one of the following mutually exclusive categories: (1) use of metformin alone and (2) use of SU drugs (alone or in combination with metformin).

2.4 Covariates

As covariates, we assessed the following known risk factors for OHCA: cardiovascular disease, use of non-antiarrhythmic QT-prolonging drugs, and use of Vaughan-Williams class 1 or 3 antiarrhythmic drugs. Presence of cardiovascular disease was based on drug proxies and

was defined as use of any of the following drugs within 6 months before the index date: beta blockers, calcium channel blockers, renin-angiotensin system inhibitors, diuretics, nitrates, antithrombotics and statins. Non-antiarrhythmic QT-prolonging drugs were defined as advised by the CredibleMeds list (www.CredibleMeds.org). Use of Vaughan-Williams antiarrhythmic drugs class 1 or 3 and/or non-cardiac QT-prolonging drugs was defined as having a drug-dispensing record within 90 days prior to index date. We used a period of 90 days before index date for Vaughan-Williams antiarrhythmic drugs class 1 or 3 and/or non-cardiac QT-prolonging drugs in order to adjust for the direct cardiac electrophysiological effects of these drugs. The cardiovascular drug groups were included in the analysis as proxies for cardiovascular disease, and not because of their direct effects on cardiac electrophysiology. Therefore, their exposure window was set at 6 months.

2.5 Cellular electrophysiological studies

The effects of the SU drugs on AP-shortening during simulated ischaemia (SI) were measured in hiPSC-CMs, a well-established human cell model for cardiac disease and drug screening studies.[8] APs of hiPSC-CMs were recorded at 36 ± 0.2°C using the perforated patch-clamp technique. APs were elicited at 1 Hz and AP duration at 90% of repolarization (APD_{90}) was analysed. The dynamic clamp technique with injection of an in silico inward rectifier K^+ current (I_{K1}) was used to achieve a close-to-physiological resting membrane potential. The effects of SU drugs on AP-shortening were studied during SI, induced by omission of extracellular glucose in combination with metabolic inhibition achieved using 2 mmol/L sodium cyanide (NaCn) and 1 mmol/L iodoacetate. An extended methods section is provided in the Supporting Information. The SU drug concentrations studied (glibenclamide 10 nM, glimepiride 10 nM, gliclazide 10 μM, tolbutamide 10 μM) were used because they are reported to cause 50% block of K_{ATP} current ($I_{K,ATP}$) in cardiac and skeletal muscle.[9-11]

2.6 Statistical analyses

We used multivariable logistic regression analysis to estimate the strength of the association between SU drugs and OHCA risk by calculating the odds ratio (OR) and 95% confidence interval (CI), employing two models. By subselecting all individuals with drug-dispensing for metformin or SU drugs 90 days before the index date from the original case–control data set, the original matching was lost. Therefore, we adjusted for age and sex (Model 1). Estimates were additionally adjusted for all covariates listed in Table 1 (Model 2). Analyses were conducted for the overall use of SU drugs (reference: metformin alone), and separately for use of SU drugs alone or in combination with metformin (reference: metformin alone). Subgroup analyses were conducted in the subgroups of OHCA cases who suffered OHCA in the presence or absence of AMI. We studied the association between individual SU drugs and OHCA risk in which the reference category consisted of glimepiride. We chose glimepiride as reference category because a previous study found that glimepiride showed no association with sudden cardiac arrest/ventricular arrhythmia.[12] Estimates for individual SU drugs were additionally adjusted for diabetes severity that was defined

according to medication prescriptions (Model 3): (stage 1) metformin or SU drugs only; (stage 2) metformin and SU drugs. Finally, we examined whether patient characteristics or concomitant drug use was different between users of individual SU drugs.

Comparisons for continuous variables were made with Student's t-test or analysis of variance (ANOVA). The χ^2 analyses were used when discrete variables were compared across groups. Statistical tests were two-tailed, with *P*-value of <.05 considered as statistically significant.

For the cellular electrophysiological studies, statistical analysis was carried out with SigmaStat 3.5 software and data are presented as mean ± SEM. Normality and equal variance assumptions were tested with the Kolmogorov–Smirnov and the Levene median test, respectively. Groups were compared using ANOVA based on ranks test (Kruskal-Wallis test) followed by Dunn's test. $P < .05$ was defined as statistical significance.

2.7 Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY, and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[13]

3 RESULTS

3.1 Subject characteristics

We identified 2503 OHCA cases from cardiac causes with ECG documented VT/VF and complete drug-dispensing records; among these cases, 219 used metformin in monotherapy, SU drugs alone or SU drugs in combination with metformin (mean age 71.4 years, 76.7% male, Table 1, Figure 1). Among 10543 non-OHCA controls with complete medication records, 697 used metformin in monotherapy, SU drugs alone or SU drugs in combination with metformin (mean age 71.9 years, 79.5% male, Figure 1).

3.2 Association between SU drugs and OHCA risk

Compared to use of metformin alone (cases: 50.2%; controls: 39.5%), the overall use of SU drugs (cases: 49.8%; controls: 60.5%) was associated with reduced risk of OHCA ($OR_{SUdrugs\text{-}overall}$ 0.6 [95% CI 0.5–0.9]). Use of SU drugs alone (cases: 19.6%; controls: 24.7%) or in combination with metformin (cases: 30.1%; controls: 35.9%) was associated with reduced OHCA risk ($OR_{SUdrugs\text{-}alone}$ 0.6 [95% CI 0.4–0.9], $OR_{SUdrugs}$ + metformin 0.6 [95% CI 0.4–0.9], Table 2). We found higher prevalence of statin use among metformin users, but found no further evidence that users of SU drugs had less cardiovascular drug use (Table 3). When we stratified according to the immediate cause of VF (AMI vs. no AMI), we found no differences in OHCA risk between SU drug users who suffered OHCA inside or outside the context of AMI ($OR_{AMI\text{-}SUdrugs}$ 0.6 [95% CI 0.4–1.1]; $OR_{nonAMI\text{-}SUdrugs}$ 0.7 [95% CI 0.7–1.2], Table 4). Next, we examined OHCA risk of

Table 1. Baseline characteristics of cases and controls

	Cases (*n* = 219)	**Controls (*n* = 697)**	***P*-value**
Mean age, years (SD)	71.4 (10.3)	71.9 (10.0)	.581
Male sex	168 (76.7)	556 (79.8)	.332
Concomitant drug use			
Beta blockers	110 (50.2)	261 (37.4)	.001
Calcium channel blockers	46 (21.0)	155 (22.2)	.700
Antithrombotics	95 (43.4)	296 (42.5)	.812
Diuretics	131 (59.8)	308 (44.2)	<.001
Renin-angiotensin system inhibitors	148 (67.6)	422 (60.5)	.061
Nitrates	54 (24.7)	78 (11.2)	<.001
Statins	129 (58.9)	450 (64.6)	.130
Vaughan-Williams class 1 or 3 antiarrhythmic drugs	14 (6.4)	10 (1.4)	<.001
Non-cardiac QT-prolonging drugs	13 (5.9)	33 (4.7)	.478

Numbers are number (%) unless indicated otherwise. *P*-values are calculated using the Student's t-test or χ^2 statistics. Use of beta blockers, calcium channel blockers, antithrombotics, diuretics, renin-angiotensin system inhibitors, nitrates and/or statins was defined as use within 6 months before the index date. Use of Vaughan-Williams class 1 or 3 antiarrhythmic drugs and/or non-cardiac QT-prolonging drugs was defined as use within 90 days before the index date.

the individual SU drugs compared to glimepiride, and found that OHCA risk was reduced in individuals who used gliclazide (OR_{adj} 0.5 [95% CI 0.3–0.9]), but not glibenclamide (OR_{adj} 1.3 [95% CI 0.6–2.7]), Table 5). Use of tolbutamide also appeared to be associated with reduced OHCA risk, but this association just failed to reach statistical significance (OR_{adj} 0.6 [95% CI: 0.3–1.002]). When we compared concomitant drug use between patients who used each of the studied SU drugs, we found no significant differences in cardiovascular drug use between individual SU drugs (Table 6).

3.3 Cellular electrophysiological studies

Figure 2A, left panels, shows typical SI-induced APD_{90} changes in time in absence (top panels) and presence (bottom panels) of 10 nM glibenclamide. In both conditions, SI resulted in an initial APD_{90} prolongation. Subsequently, APD_{90} shortened (Figure 2A), which is importantly due to activation of $I_{K,ATP}$. In presence of glibenclamide, the APD_{90} shortening was less pronounced (Figure 2A). Figure 2B, left panel, summarizes the average SI-induced APD_{90} changes in time without or in the presence of the four SU drugs. Only glibenclamide resulted in significantly less APD_{90} shortening after 15 minutes, while the other SU drugs had no significant effects (Figure 2B, right panel). Similar effects were found in a different set of experiments where we used 10 μM of all drugs (Supplemental Figure S1).

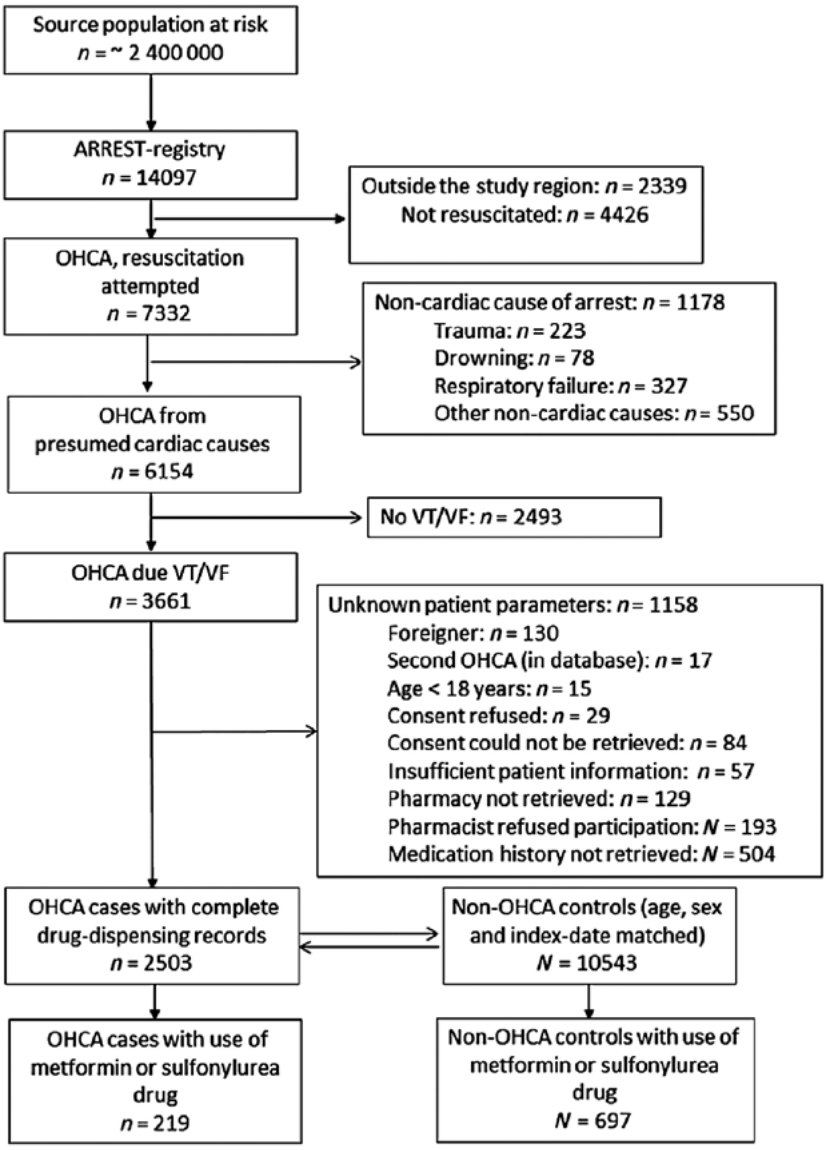

Figure 1. Flow chart of inclusion of out-of-hospital cardiac arrest (OHCA) casesOHCA, out-of-hospital cardiac arrest; VT/VF, ventricular tachycardia/ventricular fibrillation

Table 2. Use of sulfonylurea and the risk for out-of-hospital cardiac arrest compared to use of metformin in monotherapy.

	Cases (*n* = 219)	**Controls (*n* = 697)**	**Crude OR (model 1)**	**Adjusted OR (model 2)**
Metformin alone	110 (50.2)	275 (39.5)	1.0 (reference)	1.0 (reference)
Sulfonylurea drugs	109 (49.8)	422 (60.5)	0.7 (0.5–0.9)	0.6 (0.5–0.9)
Sulfonylurea drugs alone	43 (19.6)	172 (24.7)	0.6 (0.4–0.9)	0.6 (0.4–0.9)
Sulfonylurea drugs + metformin	66 (30.1)	250 (35.9)	0.7 (0.5–1.0)	0.6 (0.4–0.9)

Use of metformin and/or sulfonylurea drugs was defined as use within 90 days before the index date.
Model 1: OR adjusted for age and sex.
Model 2: OR adjusted for age, sex, use of cardiovascular drug use, Vaughan-Williams class 1 or 3 antiarrhythmic drugs and non-cardiac QT-prolonging drugs.

Table 3. Characteristics of users of metformin alone, sulfonylurea drugs alone or sulfonylurea drugs + metformin

	Metformin alone	**Sulfonylurea drugs alone**	**Sulfonylurea drugs + metformin**	***P*-value**
N	385	215	316	
Age (years, standard deviation)	69.6 (10.1)	75.2 (9.7)	72.1 (9.5)	<.001
Sex male	302 (78.4)	162 (75.3)	260 (82.3)	.146
Concomitant drug use				
Beta blockers	150 (39.0)	88 (40.9)	133 (42.1)	.696
Calcium channel blockers	80 (20.8)	53 (24.7)	68 (21.5)	.533
Antithrombotics	165 (42.9)	87 (40.5)	139 (44.0)	.720
Diuretics	185 (48.1)	101 (47.0)	153 (48.4)	.946
Renin-angiotensin system inhibitors	234 (60.8)	128 (59.5)	208 (65.8)	.254
Nitrates	52 (13.5)	37 (17.2)	43 (13.6)	.409
Statins	260 (67.5)	122 (56.7)	197 (62.3)	.029
Vaughan-Williams class 1 or 3 antiarrhythmic drugs	8 (2.1)	7 (3.3)	9 (2.8)	.654
Non-cardiac QT-prolonging drugs	24 (6.2)	10 (4.7)	12 (3.8)	.326

Numbers are number (%) unless indicated otherwise. *P*-values are calculated using ANOVA or χ^2 statistics.
Use of metformin and/or sulfonylurea drugs was defined as use within 90 days before the index date. Use of beta blockers, calcium channel blockers, antithrombotics, diuretics, renin-angiotensin system inhibitors, nitrates and/or statins was defined as use within 6 months before the index date. Use of Vaughan-Williams class 1 or 3 antiarrhythmic drugs and/or non-cardiac QT-prolonging drugs was defined as use within 90 days before the index date.

Table 4. Use of sulfonylurea drugs and out-of-hospital cardiac arrest (OHCA) risk in patients within or outside the context of acute myocardial infarction (AMI)

	Cases	Controls	Crude OR (model 1)	Adjusted OR (model 2)
OHCA-AMI	63	697		
Metformin alone	109 (49.8)	422 (60.5)	0.7 (0.5–0.9)	0.6 (0.5–0.9)
Sulfonylurea drugs	43 (19.6)	172 (24.7)	0.6 (0.4–0.9)	0.6 (0.4–0.9)
Sulfonylurea drugs alone	12 (19.0)	172 (24.7)	0.6 (0.30–1.2)	0.6 (0.3–1.2)
Sulfonylurea drugs + metformin	19 (30.2)	250 (35.9)	0.6 (0.36–1.2)	0.7 (0.4–1.2)
OHCA-no AMI	56	697		
Metformin alone	26 (46.4)	275 (39.5)	1.0 (reference)	1.0 (reference)
Sulfonylurea drugs	30 (53.6)	422 (60.5)	0.7 (0.4–1.3)	0.7 (0.4–1.2)
Sulfonylurea drugs alone	11 (19.6)	172 (24.7)	0.6 (0.3–1.3)	0.6 (0.3–1.3)
Sulfonylurea drugs + metformin	19 (33.9)	250 (35.9)	0.8 (0.4–1.5)	0.7 (0.4–1.4)

The immediate cause of OHCA could only obtained for those individuals who survived to hospital admission. Use of metformin and/or sulfonylurea drugs was defined as use within 90 days before the index-date.
Model 1: OR adjusted for age and sex.
Model 2: OR adjusted for age, sex, use of cardiovascular drugs, Vaughan-Williams class 1 or 3 antiarrhythmic drugs and non-cardiac QT-prolonging drugs.

Table 5. Use of individual sulfonylurea drugs and risk of out-of-hospital cardiac arrest compared to use of glimepiride

	Cases (*n* = 219)	Controls (*n* = 697)	Crude OR (model 1)	Adjusted OR (model 2)	Adjusted OR (model 3)
Glimepiride	58 (26.5)	164 (23.5)	1.0 (reference)	1.0 (reference)	1.0 (reference)
Glibenclamide	12 (5.5)	29 (4.2)	1.2 (0.6–2.5)	1.3 (0.6–2.7)	1.3 (0.6–2.7)
Gliclazide	16 (7.3)	101 (14.5)	0.5 (0.3–0.8)	0.5 (0.3–0.9)	0.5 (0.3–0.9)
Tolbutamide	23 (10.5)	125 (17.9)	0.5 (0.3–0.9)	0.6 (0.3–1.002)	0.6 (0.3–1.002)

Not included in the table: Three controls that used multiple sulfonylurea drugs concomitantly and no users of SU drugs. Use of individual sulfonylurea drugs was defined as use within 90 days before the index-date.
Model 1: OR adjusted for age and sex.
Model 2: OR adjusted for age, sex, use of cardiovascular drugs, Vaughan-Williams class 1 or 3 antiarrhythmic drugs and non-cardiac QT-prolonging drugs.
Model 3: OR adjusted for age, sex, use of cardiovascular drugs, Vaughan-Williams class 1 or 3 antiarrhythmic drugs, non-cardiac QT-prolonging drugs and diabetes severity.

Table 6. Characteristics of individual sulfonylurea drug users

	Glimepiride	**Glibenclamide**	**Gliclazide**	**Tolbutamide**	***P*-value**
N	222	41	117	148	
Age, years, mean (SD)	72.0 (9.6)	73.6 (9.5)	73.8 (9.5)	75.0 (10.0)	.034
Male, *n* (%)	167 (75.2)	33 (80.5)	97 (82.9)	122 (82.4)	.251
Concomitant drug use, *n* (%)					
Beta blockers	95 (42.8)	14 (34.1)	53 (45.3)	58 (39.2)	.556
Calcium channel blockers	50 (22.5)	11 (26.8)	32 (27.4)	28 (18.9)	.389
Antithrombotics	89 (40.1)	21 (51.2)	54 (46.2)	61 (41.2)	.469
Diuretics	114 (51.4)	21 (51.2)	57 (48.7)	62 (41.9)	.334
Renin-angiotensin system inhibitors	144 (64.9)	25 (61.0)	80 (68.4)	87 (58.8)	.405
Nitrates	34 (15.3)	5 (12.2)	16 (13.7)	25 (16.9)	.842
Statins	140 (63.1)	17 (41.5)	72 (61.5)	89 (60.1)	.077
Vaughan-Williams class 1 or 3 antiarrhythmic drugs	10 (4.5)	1 (2.4)	2 (1.7)	3 (2.0)	.409
Non-cardiac QT-prolonging drugs	10 (4.5)	1 (2.4)	4 (3.4)	7 (4.7)	.885

Numbers are number (%) unless indicated otherwise. *P*-values are calculated using ANOVA or χ^2 statistics.
Use of individual sulfonylurea drugs was defined as use within 90 days before the index date. Use of beta blockers, calcium channel blockers, antithrombotics, diuretics, renin-angiotensin system inhibitors, nitrates and/or statins was defined as use within 6 months before the index date. Use of Vaughan-Williams class 1 or 3 antiarrhythmic drugs and/or non-cardiac QT-prolonging drugs was defined as use within 90 days before the index date.

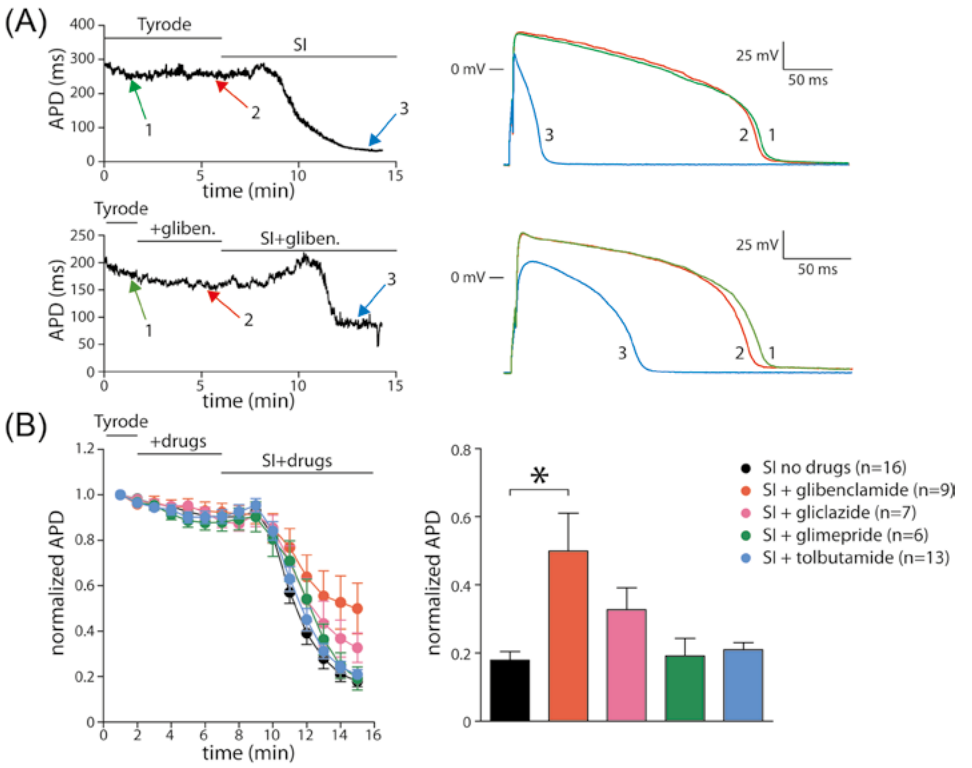

Figure 2. Effects of sulfonylurea drugs on action potentials (APs) during simulated ischemia (SI). A, Typical AP duration (APD) changes in time in response to SI in absence **(top panels)** or presence **(bottom panels)** of 10 nM glibenclamide. SI-induced APD shortening is less pronounced in presence of glibenclamide. **B**, Average SI-induced APD changes in time **(left panel)** and average APD shortening at 15 min in absence or presence of glibenclamide (10 nM), gliclazide (10 μM), glimepride (10 nM), and tolbutamide (10 μM). * indicates $P < .05$

4 DISCUSSION

In this observational study using real-world population-based data, we found that use of SU drugs (alone or in combination with metformin) was associated with decreased OHCA risk compared to use of metformin alone. Although users of metformin were younger and the prevalence of statin use was higher among metformin users, we found no further evidence that differences in patient profiles between SU drug users and metformin users accounted for the OHCA risk-reducing effects of SU drugs. There was no difference in OHCA risk reduction between patients with or without AMI. Finally, we found that gliclazide, but not glibenclamide, was associated with decreased OHCA risk compared to glimepiride; tolbutamide also appeared to be associated with reduced OHCA risk, but this association just failed to reach statistical significance. The differences between the SU drugs were not explained by differences in patient characteristics or by different effects on APD_{90} changes during simulated ischaemia in cellular electrophysiologic studies in hiPSc-CMs.

To find the explanation for the decreased risk of OHCA upon use of SU drugs, we first investigated whether our finding was explained by differences in patient profiles, in particular, presence of factors that increase OHCA risk (i.e., cardiovascular comorbidity). Although the prevalence of statin use was higher among users of metformin, we found no further evidence that users of SU drugs had fewer prescriptions for cardiovascular drugs than users of metformin. Second, since diabetes itself is a known risk factor for OHCA, confounding by indication must be considered.[2] Such confounding by indication may arise when comparing treatments at different stages of the disease process (time-lag bias). In our study, we therefore compared use of SU drugs as a whole and separately as monotherapy and in combination with metformin, to use of metformin in monotherapy. Even when we compared use of SU drugs in combination with metformin (a second- to third-line treatment strategy) to metformin monotherapy (a first-line treatment strategy), use of SU drugs was associated with decreased OHCA risk. Furthermore, patients using SU drugs (with or without metformin) might have more diabetes severity than patients using metformin only (our reference category). Nonetheless, despite probably having more advanced stage of diabetes than users of metformin only, users of SU drugs still had lower OHCA risk; this provides additional support for the notion that SU drugs reduce OHCA risk.

Since SU drugs are usually prescribed in addition to metformin as a second- to third-line treatment strategy, individuals had to survive to receive SU drugs. Thus, individuals who only used metformin in the 90 days prior to index date and an OHCA occurred were classified in the metformin group. However, individuals who subsequently received SU drugs in the 90 days prior to index date would have been classified in the SU drugs + metformin group. This may result in immortal bias, because the period during which individuals were on metformin and survived (immortal) was not counted (in the metformin group) in the calculation of the effect size.

We found no stronger OHCA risk reduction associated with SU drugs in OHCA patients with proven AMI than in OHCA patients without proven AMI. This does not rule out that the OHCA risk-reducing effects of SU drugs occur in the presence of myocardial ischaemia, because myocardial ischaemia may have been present, but may not have led to AMI, e.g., because of spontaneous reperfusion by thrombus resolution and/or relaxation of the culprit vessel.[14] This is of particular relevance, since AMI status could only be established in OHCA victims who survived to hospital admission and received cardiologic workup (post-mortem analysis after OHCA is not mandatory in the Netherlands and very seldom performed in OHCA victims who die before hospital admission).

We next studied the effects of each individual SU drug separately considering that SU drugs differ substantially between each other in several important pharmacodynamic and pharmacokinetic properties.[5] Our findings indicate that the OHCA risk-reducing effects of SU drugs are drug-specific, rather than a class effect. We investigated whether they were explained by differences in patient profiles, but found no relevant differences in concomitant drug use between users of the different SU drugs. In addition, we studied whether the SU drugs have distinct potencies to

inhibit K_{ATP} channels during SI. We found that this was the case, with glibenclamide preventing AP-shortening during SI more than the other SU drugs studied, including glimepiride. However, these findings were not consistent with our epidemiologic finding that the SU drugs were grouped into two groups: drugs that reduced OHCA risk (gliclazide, tolbutamide, although not statistically significant for tolbutamide) and drugs that did not (glibenclamide, glimepiride). Thus, our cellular electrophysiologic studies provided no evidence to support the possibility that our observed effects of SU drugs were explained by their effects on APD shortening during ischaemia.

Although we found an association between SU drugs and reduced OHCA risk, it is important to note that some guidelines have already lowered the recommendation for the use of SU drugs[15] due to their association with increased risk of hypoglycaemia[16] and the increasing concerns regarding their cardiovascular safety.[17, 18] Compared to newer oral antidiabetic drugs, SU drugs are associated with higher risk of hypoglycaemia. A recent study reported that hypoglycaemic adverse events occurred in 10.6% of linagliptin users, but in 37.7% of glimepiride users.[19] Still, given the low cost and demonstrated ability to reduce microvascular complications, SU drugs remain important in the treatment of diabetes mellitus type 2.[20] SU drugs may differ with respect to hypoglycaemia risk with glibenclamide carrying the highest and gliclazide the lowest risk of hypoglycaemia among second-generation SU drugs, which may also influence OHCA risk, since hypoglycaemia has been associated with QT-prolongation.[12] This may have influenced our outcome, since we could not control for hypoglycaemia in our study. This may have masked a possible association between glibenclamide and reduced OHCA risk, while we expect that the association between gliclazide and OHCA may have been less affected.

A previous study showed an increased mortality and cardiovascular risk with most first- and second-generation SU drugs compared with metformin in patients with or without previous AMI.[17] However, results were not statistically different from metformin in both patients with AMI and those without AMI for gliclazide,[17] indicating that gliclazide has a more favourable cardiovascular risk profile over other SU drugs. These findings were supported by another retrospective cohort study, where an increased total and cardiovascular mortality associated with glibenclamide compared with gliclazide was found.[18] Our finding that gliclazide was associated with lower OHCA risk adds to the accumulating evidence of a favourable cardiovascular risk profile for gliclazide over other SU drugs. Regardless of the underlying mechanisms of our epidemiologic findings, our results are of clinical importance given the sharp rise in the prevalence of diabetes, and the fact that diabetes is associated with increased OHCA risk.[2] Therefore, a potential relation between gliclazide and lower OHCA risk and the mechanisms involved warrants future replication studies in other settings.

Animal studies on the effect of K_{ATP} channel modulators on cardiac arrhythmias produced seemingly contradictory findings. Glibenclamide was associated with decreased incidence of sustained VT and VF during ischaemia–reperfusion,[21] but another study found increased

occurrence of VT.[22] Conversely, use of K_{ATP} channel openers was associated with increased incidence of VT/VF during ischaemia–reperfusion in one study,[21] but with decreased incidence of arrhythmias in another study.[23] Moreover, opposite effects in the propensity for VF of K_{ATP} channel openers were demonstrated in different species.[23] While these drugs inhibited VF in anesthetized dogs, they promoted VF in rat hearts. These discrepancies indicate that the effects of K_{ATP} channel modulators in relation to cardiac arrhythmias depend upon the experimental model.[19] The effects of SU drugs on VT/VF on a population level are less known, and previous clinical studies on the association between SU drugs and VT/VF had important limitations.[12, 24-26] A retrospective study by Davis et al. showed that glibenclamide, but not gliclazide, was associated with lower VF incidence compared to insulin, but not to gliclazide.[24] However, important facts were not reported, e.g., the time of VF occurrence, the temporal relationship between VF and SU drug use, and the number of VF cases. Also, the diagnosis of diabetes partly relied on self-reported data, and VF ascertainment was not systematic, e.g., fewer diabetes patients received rhythm monitoring.[24] Lomuscio et al. found lower VF incidence in the first 36 hours after AMI among individuals with diabetes who used glibenclamide compared with individuals with diabetes who used no glibenclamide.[25] That study, however, was small (VF occurred in two individuals with diabetes who used glibenclamide, and six individuals with diabetes who did not use glibenclamide), while VT/VF episodes occurred at an unspecified time during the 36 hour period (and may not have been related to $I_{K\text{-}ATP}$ activity), and it was not specified whether all patients used glibenclamide at the time of those episodes. Cacciapuoti et al. reported that premature ventricular complexes and nonsustained ventricular tachycardias following presumed ischaemic episodes occurred less often during glibenclamide use than metformin use in a crossover study.[26] However, the study was small (19 individuals with diabetes), and ascertainment of ischaemia was uncertain, relying solely on ST segment changes on Holter recordings with an unspecified number of leads.[26] Leonard et al. investigated in a large cohort (519 272 patients) whether SU drugs are associated with risk of sudden cardiac arrest and ventricular arrhythmia, and found reduced risk among users of glibenclamide, but not glimepiride, compared with glipizide (tolbutamide and gliclazide were not examined).[12] We could not compare our findings with that study, because glipizide is not marketed in the Netherlands, while gliclazide is not marketed in the United States and in all European countries. Moreover, the study populations are distinct, with far larger proportions of women and non-Caucasians among study participants in that study than in ours. In addition, the study by Leonard et al. had important limitations. First, that study relied solely on emergency department and in-hospital diagnosis, and omitted patients who died before hospital admission. This causes important inclusion bias, particularly since diabetes is associated with reduced pre-hospital survival rates after OHCA.[27] Moreover, ascertainment of VF was not certain because it was based on claims rather than actual ECG recordings. Our study resolved these limitations. Our ARREST registry was specifically designed to study OHCA and, thanks to participation of all EMS departments in the study region, enrolled both patients who survived to hospital admission and those who died pre-hospital. Moreover, all cases in the ARREST registry had ECG documentation to ascertain the presence or absence of VT/VF.

4.1 Strengths and limitations

A major strength of the ARREST registry is the presence of ECG documentation of VT/VF. Given the highly unpredictable way in which OHCA occurs and the low survival rates after OHCA, it is very difficult to study OHCA. Such studies require a dedicated study design, in particular, to ascertain that OHCA resulted from cardiac causes and to reduce misclassification by inclusion of OHCAs from non-cardiac causes. ECG documentation of VT/VF may be the best method to achieve this. Moreover, the population-based real-world design of our ARREST registry minimizes selection bias by ensuring that virtually all OHCAs in our study region are prospectively captured. This is particularly relevant in the present study, since patients with diabetes have reduced pre-hospital survival rates after OHCA.[23] Finally, information about drug use in cases and controls was based on drug-dispensing records, which is already one step closer to ascertainment of use than analysis of drug prescription.

Our study has also some limitations, e.g., data regarding the comorbidities could not be included in this study. To deal with this, we used concomitant drug use as proxy. Although we have no direct evidence that this method captures the most important cardiovascular diseases sufficiently accurately, we derive assurance that it did from our previous study in which we found similar results when we used this method or direct information on comorbidities.[28] Moreover, possible misclassification arising from this was probably similarly distributed between cases and controls. Furthermore, possible confounding by indication might play a role in our study since diabetes itself is a known risk factor for OHCA.[2] An approach could be to match case and control subjects on duration of diabetes. Here, duration of antidiabetic treatment might be considered as a proxy for diabetes duration. However, the fact that we only had information on medication use 1 year before index year prevented us from identifying disease duration in all patients. To minimize this potential bias, we selected only patients with diabetes, aiming to make the cases and controls comparable with respect to underlying condition and excluded patients using second-line antidiabetic drugs or insulin to create a study population with a similar disease severity. Moreover, we compared users of SU drugs to users of metformin only. Still, (unmeasured) residual confounders could not be ruled out since data on several important risk factors such as physical activity, body mass index, left ventricular ejection fraction and blood glucose regulation were not available. Similarly, we had no information about smoking and alcohol use, although we cannot rule out that these confounders were unequally distributed between cases and controls, thereby leading to different baseline risk of cardiovascular disease between both groups. Also, we had no information about renal function and were not able to adjust for it. Yet, the risk of OHCA increases as kidney function declines.[29] Socioeconomic position is another risk factor for which we could not adjust because of lack of data, but it is of possible relevance, as it is likely associated with OHCA incidence.[30] Finally, misclassification in the use of metformin and SU drugs may have occurred, as we defined drug use as presence of drug-dispensing records ≤90 days prior to index date. We considered an exposure window of 90 days, since, in the Netherlands, the average repeat prescription length for drugs used for chronic diseases is 90 days. Lengthening of this exposure window might lead to

inclusion of drugs that were not used at index date, confounding the analysis of direct drug effects on the primary endpoint of OHCA, as we planned in the present study. Lastly, patients without a proven immediate cause of OHCA were excluded from the analysis that was stratified according to AMI status. Yet, exposure and AMI status are unrelated to missingness, and therefore we do not expect that the exclusion of cases with missing AMI status has impacted our results. Our subgroup analysis was based on small sample sizes, which may have resulted in possibly low statistical power. Thus, findings regarding our subgroup analyses should be interpreted with caution.

5 CONCLUSION

SU drugs (alone or in combination with metformin) were associated with reduced OHCA risk compared to metformin monotherapy. Gliclazide was associated with lower risk than glimepiride. These effects were not solely explained by effects on AP-shortening during ischaemia.

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COMPETING INTERESTS

There are no competing interests to declare.

CONTRIBUTORS

T.E.E. and H.L.T. conceived the study idea. T.E.E., M.T.B., A.O.V., P.C.S., A.d.B., H.L.T. designed the research. T.E.E. performed the statistical analyses and wrote the manuscript; A.O.V. and L.J. conducted the patch-clamp experiments. P.C.S. worked up the original data to a data matrix ready for statistical analyses. All authors critically revised and approved the manuscript.

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SUPPLEMENTARY MATERIAL

Methods

Electrophysiology

Supplemental Table 1. Anatomical Therapeutic Chemical classification (ATC) system for the sulfonylurea drugs

Results

Supplemental Figure 1. Effects of 10 μM sulfonylurea drugs on action potentials (APs) during simulated ischemia (SI). Left panel, Average changes over time in AP duration (APD) induced by SI in absence or presence of 10 μM sulfonylurea drugs. Right panel, Average APD shortening at 15 min after start of SI in absence or presence of 10 μM sulfonylureas. * indicates P<0.05

Methods Electrophysiology

Cell preparation

The effects of the four sulfonylureas on cardiac function were measured in human induced pluripotent stem cell (hiPSC) derived cardiomyocytes (hiPSC-CMs), a well-established human cell source for cardiac diseases modeling and drug screening.[1, 2] hiPSC-CMs were generated from the control hiPSC line, LUMC0099iCTRL04, which was derived from skin fibroblasts of a Caucasian woman, using a mRNA based reprogramming method.[3] The LUMC0099iCTRL04 line is registered in the Human Pluripotent Stem Cell Registry (hPSCreg), that contains all details pertaining to its generation and characterization.[4] Differentiation to CMs was performed using BPEL medium[5] containing Activin-A (Miltenyi Biotec), BMP4 (R&D Systems), and CHIR99021 (Axon Medchem) as reported previously.[6] After 3 days, the medium was replaced by BPEL containing XAV939 (Tocris Biosciences) until day 7. Typically, contractile CMs were observed from day 12 onwards. hiPSC-CMs were dissociated at day (d)17 using TrypLE Select (Life Technologies), and plated at a low density (~$7.5x10^4$ cells) on Matrigel-coated coverslips in BPEL medium. Medium was refreshed every 3-4 days and electrophysiological measurements were performed in single hiPSC-CMs 7-9 days after dissociation.

Data acquisition

Action potentials (APs) were recorded at 36 ± 0.2°C using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data acquisition and analysis were performed with custom software. Signals were low-pass-filtered with a cutoff of 5 kHz and digitized at 40 kHz, and the potentials were corrected for the calculated liquid junction potentials.[7] Cell membrane capacitance (C_m) was calculated by dividing the time constant of the decay of the capacitive transient after a −5 mV voltage step from −40 mV by the series resistance

Action potential recordings

APs were measured in hiPSC-CMs using the perforated patch-clamp technique. hiPSC-CMs have a small or even absent inward rectifying K^+ current (I_{K1}).[8] Consequently, hiPSC-CMs

have a depolarized maximal diastolic potential and are frequently spontaneously active.[1] We overcame this limitation by injection of an *in silico* 2 pA/pF I_{K1} with kinetics of Kir2.1 channels through dynamic clamp,[8] which resulted in close-to-physiological resting membrane potentials (RMPs). Pipettes (resistance 2–3 MΩ; Harvard Apparatus, UK) were filled with solution containing (in mmol/L): 125 K-gluconate, 20 KCl, 5 NaCl, 0.44 amphotericin-B, 10 HEPES; pH 7.2 (KOH). The baseline bath solution was Tyrode's solution containing (in mM): 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5.5 glucose, 5.0 HEPES; pH 7.4 (NaOH). Simulated ischemia shortens the cardiac APs due to opening of ATP-regulated K^+ channels.[9] The effects of sulfonylureas on AP shortening were studied during simulated ischemia, which was instituted by omission of glucose in combination with metabolic inhibition achieved using 2 mmol/L sodium cyanide (NaCn) and 1 mmol/L iodoacetate.[9] APs were elicited at 1 Hz by 3-ms, ~1.2´ threshold current pulses through the patch pipette, and AP duration at 90% of repolarization (APD_{90}) was analyzed.

Drugs

NaCn and iodoactetate were dissolved in the simulated ischemia solution directly. Glibenclamide, gliclazide, glimepiride, and tolbutamide were freshly prepared as stock solutions in DMSO and all stock solutions were >1000´ diluted before use. All drugs were obtained from Sigma-Aldrich (St.Louis, MO, US), except NaCN which was from Merck (Darmstadt, Germany).

Statistics

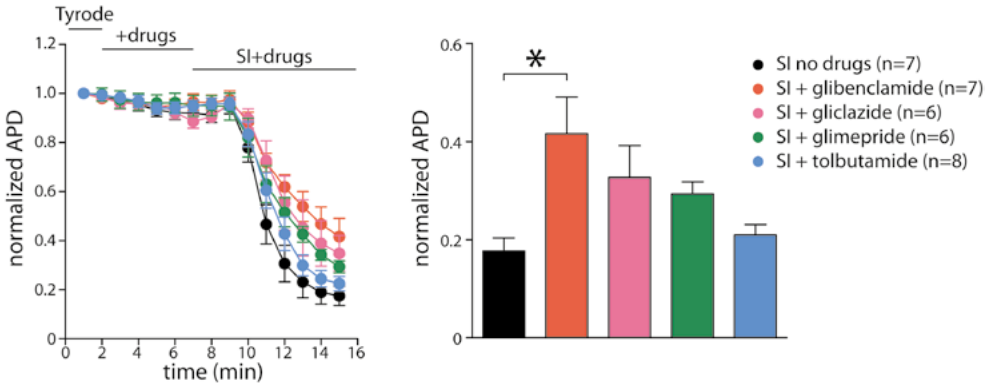
Statistical analysis was carried out with SigmaStat 3.5 software and data are presented as mean±SEM. Normality and equal variance assumptions were tested with the Kolmogorov-Smirnov and the Levene median test, respectively. Groups were compared using ANOVA based on ranks test (Kruskal-Wallis test) followed by Dunn's test. $P<0.05$ was defined as statistical significance.

Supplemental Table 1. ATC codes of sulfonylurea drugs

Sulfonylurea drugs	A10BB, A10BD02, A10BD04
Glibenclamide	A10BB01, A10BD02
Glimepiride	A10BB12, A10BD04
Gliclazide	A10BB09
Tolbutamide	A10BB03

Results

Electrophysiology



Supplemental Figure 1. Effects of 10 μM sulfonylurea drugs on action potentials (APs) during simulated ischemia (SI). Left panel, Average changes over time in AP duration (APD) induced by SI in absence or presence of 10 μM sulfonylurea drugs. **Right panel**, Average APD shortening at 15 min after start of SI in absence or presence of 10 μM sulfonylureas. * indicates P<0.05

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CHAPTER 6

GENERAL DISCUSSION AND PERSPECTIVE

OVERVIEW

Sudden cardiac arrest (SCA) is a worldwide public health problem, and responsible for ≈50% of the mortality from cardiovascular disease in the United States and other developed countries, and for ≈20% of mortality in the general population.[1-3] To develop and evaluate preventive strategies, the causes and mechanisms of SCA are studied by many scientists. Drugs may facilitate the occurrence of life-threatening cardiac arrhythmias and SCA; this receives increasing attention from medical experts. The mechanism whereby drugs may impact on SCA risk can be evaluated using both *in vivo* and *in vitro* tests. Assessing the ability of chemical molecules to modify ion channels via single-cell electrophysiological studies is widely used in preclinical studies. This thesis takes the observed effects on SCA risk in the population of some drugs as a basis to investigate and uncover the potential mechanisms by which these medicines might induce SCA at the cellular level. Several noncardiac drugs acting on the neurological system have been linked to cardiac electrophysiological changes, as evidenced by QT prolongation or Brugada Syndrome (BrS) pattern in the ECG, both of which are linked to SCA risk.[4, 5] Anti-epileptic drugs (AEDs) and opioids are two groups of such drugs which were studied in this thesis. I evaluated whether AEDs and opioids are associated with SCA risk and elucidated the possible causative mechanisms on cardiac ion channels.

Some AEDs are associated with the occurrence of the BrS ECG pattern, suggesting that these drugs inhibit inward current such as Na^+ current (I_{Na}) or L-type Ca^{2+} current ($I_{Ca,L}$).[6, 7] In **chapter 2**, I investigated whether the AED carbamazepine is associated with an elevated risk of SCA in a large dataset from a cohort especially designed to study SCA in the general population, and used patch-clamp methods to determine the effects of CBZ on action potentials (APs) and individual membrane currents of rabbit and human cardiomyocytes. I found that CBZ use is associated with increased SCA risk in the general population, which is consistent with a previous report.[8] However, the findings from this epidemiological analysis should be interpreted with caution due to the confounding effect of epilepsy, which is associated with increased SCA risk.[9] Subsequently, I conducted *in vitro* measurements on the effects of multiple concentrations of CBZ (1 to 100 μM), including concentrations corresponding to therapeutic plasma concentrations. I found that CBZ reduces cardiac AP upstroke velocity (dV/dt_{max}) and I_{Na} in human and rabbit cardiomyocytes and causes a predisposition to (in rabbits) and considerable (in humans) AP shortening and $I_{Ca,L}$ reduction, while leaving K^+ currents unaffected. All of the results are consistent with each other: reduced AP upstroke velocity is well explained by reduction in I_{Na}.[10]

In chapter 3, I studied five other AEDs which, next to carbamazepine, have the largest number of users in the Netherlands, i.e., gabapentin, lamotrigine, levetiracetam, pregabalin, and valproic acid. In this chapter, I conducted patch-clamp studies to evaluate the effects of these drugs on densities and gating properties of human cardiac Na^+ (Nav1.5) channels stably expressed in a HEK293 cell line, and on AP properties of freshly isolated rabbit cardiomyocytes. I found that lamotrigine and valproic acid inhibited the Nav1.5 current in a dose-dependent manner,

while gabapentin, levetiracetam, and pregabalin had no effect on Nav1.5 current amplitudes at the doses tested. In addition, lamotrigine and valproic acid induced a hyperpolarizing shift of steady-state inactivation, and a delay in recovery from inactivation. The changes in Nav1.5 current properties were reflected by a reduction in dV/dt_{max} and AP amplitude (APA) in rabbit cardiomyocytes. The antagonizing effects of (100 μM) lamotrigine and (3000 μM) valproic acid on the Nav1.5 current, dV/dt_{max}, and APA are here reported for concentrations that exceed the upper limit of the therapeutic range of lamotrigine (59 μM) and valproic acid (867 μM),[11, 12] but only by a factor of 2–3 (while a reduction in Nav1.5 current density by lamotrigine already started at concentrations within its therapeutic range). As a result, these effects may be clinically significant because these slightly higher concentrations may occur in clinical practice. To summarize chapter 2 and 3, our results demonstrate a plausible mechanism by which these drugs exert a proarrhythmic effect, which could contribute to increase the risk of SCA.

Opioids, while targeting the nervous system, have also been implicated in the prolongation of the QT interval of the ECG.[13] This proclivity is attributable to the effect of inhibiting outward K^+ currents, especially the rapid component of the delayed rectifier K^+ current (I_{Kr}) [14, 15] However, the opioids oxycodone and methadone were demonstrated to inhibit also Nav1.5 channels.[16, 17] Therefore, **in chapter 4**, my aim was to verify if other opioids, i.e., tramadol, codeine, and fentanyl, could also block Nav1.5 current, which may be one mechanism to increase the risk of SCA. Tramadol reduced Nav1.5 current expressed in HEK293 cells with an increased affinity in Nav1.5 channels that were partially in a state of fast inactivation or slow inactivation. Of note, compared to the IC_{50} of tramadol for blocking Nav1.5 current in their fully-available state, the IC_{50} of partially fast-inactivated or slow-inactivated Nav1.5 is more relevant for extrapolation to the physiological condition, since at the normal resting membrane potential (RMP), a substantial proportion of Nav1.5 channels are inactivated. Apart from reducing Nav1.5 current density, tramadol also slowed its recovery from inactivation. Tramadol reduced dV/dt_{max} and APA in rabbit ventricular cardiomyocytes with a larger amount of reduction at fast pacing rates. Tramadol caused no significant changes in AP duration at 90% of repolarization (APD_{90}).

In chapter 5, I report a protective effect of noncardiac drugs in the specific condition of acute myocardial ischemia and infarction. Sulfonylurea drugs, used commonly to achieve glycaemic control in diabetes, exert their therapeutic action by blocking pancreatic ATP-sensitive K^+ (K-ATP) channels, thereby inducing release of insulin. SU drugs may also block myocardial K-ATP channels,[18] thereby potentially impacting on AP duration of ventricular cardiomyocytes. In this chapter, we found that use of these drugs is associated with reduced risk of SCA in patients with diabetes. We hypothesized that these medications could reduce the incidence of SCA by counteracting the shortening of the AP duration that results from K_{ATP} channel activation during myocardial ischemia. The effects of the SU drugs glibenclamide, gliclazide, glimepride, and tolbutamide on APD_{90} changes induced by simulated ischemia were tested using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Glibenclamide attenuated simulated ischemia-induced APD_{90} shortening, while the other SU drugs had no effect. SU drugs

were associated with reduced SCA risk compared to metformin monotherapy, with gliclazide being associated with a lower risk than glimepiride. However, these differential epidemiological effects of the SU drugs were not reflected by the patch-clamp data. Unraveling the mechanism of the protective effect of SUs on a population level requires further study.

INTERPRETATION OF CELL MODELS

In this thesis, we used various cell models, including rabbit ventricular cardiomyocytes, human atrial cardiomyocytes, HEK293 cells, and hiPSC-CMs. All of these cell models have their benefits and limitations. For instance, the freshly isolated human cardiomyocyte is the best cell model to study drug effects and to extrapolate the laboratory data to clinical practice. However, human hearts and tissue are not frequently available and, in addition, it is quite difficult to isolate viable cells from this tissue.[19] In contrast, hearts and enzymatically isolated cardiomyocytes of rabbits are more easily available, and the AP morphology and set of their ion channels compares well to human cardiomyocytes,[20, 21] so it is an often used animal cell models for *in vitro* electrophysiological studies. **In chapter 2**, we used rabbit ventricular cardiomyocytes to study the cardiac electrophysiological effects of CBZ. The principal findings in these cells were also confirmed in human atrial myocytes, thus making our data more convincing. The HEK293 cell is a frequently used cell expression system used to study the biophysical properties of ion channels.[20, 22] It is an excellent cell model for studying single ion channels while avoiding interference from other ion channels. However, one obvious limitation of this cell model is that it cannot generate APs because it contains a single ion channel type, while the generation of an AP requires the concerted activity of multiple ion channels and ion transporters. **In chapters 3** and **4**, we used HEK293 cells with stable Nav1.5 current expression[23] to study the effects of AEDs and opioids on Nav1.5 current properties. In addition, we studied the changes in AP properties that these drugs induced in rabbit ventricular cardiomyocytes. **In chapter 5**, we used hiPSC-CMs to study the effects of SU drugs in a simulated ischemia condition. hiPSC-CMs, a human-derived cell line, display many essential features of human cardiomyocytes, such as the expression of functional proteins, e.g., ion channels. The use of hiPSC-CMs has rapidly developed in recent years.[24] This cell model can be functionally characterized *in vitro* to model the complex cellular physiology of cardiomyocytes, and theoretically provide an unlimited supply of human cardiomyocytes.[25-27] However, it also has some obvious limitations. For instance, compared to adult ventricular cardiomyocytes, in terms of cardiac ion channel functional availability, hiPSC-CMs show notably lower inward rectifier K^+ current (I_{K1}) density.[28] Low I_{K1} density-induced depolarization of the RMP could lead to the loss of function of individual membrane currents to some extent due to partial inactivation, thereby reducing their availability.[28] To solve this problem, we used dynamic clamp by injection of an I_{K1}-like current to obtain quiescent hiPSC-CMs with a nondepolarized RMP.[28]

INTERPRETATION OF PLASMA CONCENTRATIONS

An important issue when extrapolating results from *in vitro* electrophysiological studies to the clinical environment is that the used concentrations of the compounds must be comparable to concentrations found in clinical practice. When choosing drug concentrations in our studies, we based our choice on therapeutic concentrations of the drug to study the concentration-dependency of the drug effects, including the IC_{50} of drug-induced ion channel block. In chapter 4, we also used lethal drug concentrations in our initial drug screen, reasoning that drugs in which no effects were found even at lethal concentrations would not need to be studied further in more detailed analyses, because such analyses would lack clinical relevance.

In **chapter 2**, Carbamazepine was first measured at different concentrations (1–100 μM) *in vitro*, including concentrations corresponding to plasma levels that provide anticonvulsant effects (20–40 μM). Carbamazepine reduced the dV/dt_{max} of rabbit cardiomyocytes and the magnitude of I_{Na} at ≥30 μM. Meanwhile, we found a mild reduction of the depolarizing current $I_{Ca,L}$ at 100 μM. In view of the fact that these effects were observed at concentrations which corresponded to reported therapeutic plasma concentrations, we concluded from this study that reduction of Nav1.5 current may be a mechanism that contributes to the increased SCA risk associated with carbamazepine as observed in epidemiological studies. We studied gabapentin, lamotrigine, levetiracetam, pregabalin, and valproic acid in **chapter 3,** and found that lamotrigine and valproic acid inhibited Nav1.5 current in a concentration-dependent manner. The IC_{50} of these two AEDs were 142 ± 36 and 2022 ± 25 μM, respectively. The antagonistic effects of lamotrigine and valproic acid on Nav1.5 current were greater than their therapeutic concentration (lamotrigine at 59 μM[12] [10] and valproic acid at 867 μM[11] [23]), but only by a factor of 2-3. Thus, these effects may be clinically relevant, for instance, at (moderate) overdoses. Moreover, this detrimental effect may be more pronounced in vulnerable individuals, such as patients who carry loss-of-function inducing variants in genes that encode subunits of Nav1.5 channel, or under clinical conditions that are associated with reduced Nav1.5 availability, in particular, myocardial ischemia/infarction. The opioid tramadol induced an enhanced reduction of Nav1.5 current at more depolarized RMPs in **chapter 4**. The IC_{50} of tramadol for block of Nav1.5 current was 378.5 ± 33.2 μM when these channels were fully-available. Yet, the blocking effects occurred at drastically lower concentrations when these channels were in a partially fast-incativated state (4.5 ± 1.1 μM) or in a partially slow-inactivated state (16 ± 4.8 μM). We used a significantly hyperpolarized holding potential of –120 mV to study the effect of tramadol on fully-available Nav1.5 channels. While this approach is commonly employed in electrophysiological research, it may limit extrapolation to physiological settings, where the RMP of working cardiomyocytes is around –80 mV. At this potential, a significant proportion of Nav1.5 channels are in a fast-inactived state. To mimic this state, we set the holding potential at –90 mV. In addition, we measured the effect of tramadol on APs in rabbit ventricular cardiomyocytes. We found that, with increasing tramadol concentrations, dV/dt_{max} was increasingly reduced, becoming statistically significantly smaller than baseline from 100 μM onwards. These findings support

our conclusion that tramadol inhibits the Nav1.5 current even within the therapeutic range of this drug.

INTERACTION WITH NON-DRUG RISK FACTORS

The clinically observed incidence of SCA is determined by multiple risk factors that may exist in a single individual. Such risk factors include advanced age, female sex (in the case of QT-prolonging drugs), electrolyte disorders, renal or hepatic dysfunction, concurrent use of other QT-prolonging medications (in the case of I_{Kr}-blocking drugs) or drugs that induce the BrS-ECG (in the case of I_{Na}-blocking drugs) or comorbidities.[29, 30] In the case of I_{Na}-blocking drugs, relevant comorbidities are those that are associated with reduced Nav1.5 function, such as acute myocardial ischemia and infarction.[31] Enhanced vulnerability may also stem from inherited conditions, in particular, carrying variations in genes that encode subunits of the Nav1.5 channel, in particular, *SCN5A*. Loss-of-function mutations in this gene cause the inherited cardiac arrhythmia syndromes BrS[32] and cardiac conduction disease,[33] both of which are associated with an increased risk of SCA. We tested the effect of AEDs and opioids on Nav1.5 current in this thesis, and found carbamazepine, lamotrigine, valproic acid, and tramadol block the Nav1.5 channel. These results provide a rationale for clinical guidelines which recommend that, in patients with ischemia or infarction and/or those that carry loss-of-function variants in *SCN5A* or other Nav1.5-encoding genes, these drugs should be avoided or used only when stringent monitoring is applied.

PROSPECTIVE DEVELOPMENT SUGGESTIONS

Patients planning to take Nav1.5 channel blocking drugs should be examined for pertinent clinical and inherited risk factors to minimize the risk of SCA.[34] The use of drugs that potentially exert proarrhythmic side effects in patients with pre-existing risk factors should be avoided or only conducted when appropriate precautions are taken. When designing guidelines for the use of these drugs, lessons can be learned from the use of QT-prolonging (i.e., I_{Kr}-blocking) drugs. For those drugs, the American Heart Association/American College of Cardiology suggest that ECG recordings before and 8-12 hours after their initiation or dose increase should be performed to reduce the risk of developing TdP.[30] Meanwhile, medical experts (physicians, pharmacists) should be aware of medications that (inadvertently) block I_{Na} or I_{Kr}, thereby increasing SCA risk. Websites such as http://www.brugadadrugs.org and https://www.crediblemeds.org are particularly beneficial to provide this information and thereby promote this type of personalized medicine.

CONCLUSIONS

AEDs (carbamazepine, lamotrigine and valproic acid) and opioids (tramadol) reduce Nav1.5 current by reducing its current density and changing its gating properties; these effects are reflected by changes of AP properties. These novel insights may help to design clinical guidelines that aim to lead to personalized and safer use of these drugs. For instance, in individuals with

known related risk factors, such as those with acute myocardial infarction or myocardial ischemia, these drugs should be prescribed only after taking appropriate precautions or they should be avoided altogether.

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CHAPTER 7

SUMMARY / SAMENVATTING

ENGLISH SUMMARY

Sudden cardiac arrest (SCA) is a worldwide public health problem and is the main cause of death from cardiovascular disease. Drugs, including noncardiac drugs (drugs to treat noncardiac conditions), may impact on the risk of the occurrence of SCA. This may be due to the ability of these drugs to modify the cardiac ion channels whose concerted activity underlies the cardiac action potential and the heart's electrical properties.

Anti-epileptic drugs are studied in **chapters 2 and 3**. We assessed the risk of SCA caused by carbamazepine and established the possible underlying cellular electrophysiological mechanisms in **chapter 2**. The effects of carbamazepine (1–100 μM) on action potentials and individual membrane currents were determined in isolated rabbit and human cardiomyocytes using the whole-cell patch-clamp technique. Carbamazepine reduced the action potential upstroke velocity of both rabbit and human cardiomyocytes, without prominent changes in other action potential parameters. Carbamazepine (≥30 μM) also reduced the magnitude of the current of the cardiac sodium current channel (Nav1.5), and induced a hyperpolarizing shift in its voltage-dependency of inactivation, while slowing its recovery from inactivation. The cardiac L-type calcium current $I_{Ca,L}$ was reduced by 100 μM carbamazepine. In **chapter 3** the effects of five other often used anti-epileptic drugs (gabapentin, lamotrigine, levetiracetam, pregabalin, and valproic acid), which are associated with increased risk of SCA, were tested. Using cell cultures (HEK293 cells) with stable Nav1.5 expression, it was found that lamotrigine and valproic acid reduced human Nav1.5 channel currents and induced a hyperpolarizing shift of steady-state inactivation, and a delay in recovery from inactivation. The changes in Nav1.5 properties were reflected by a reduction in action potential upstroke velocity and action potential amplitude. Meanwhile, gabapentin, levetiracetam, and pregabalin had no effect on Nav1.5 current.

In **chapter 4**, we tested the effects of the opioids tramadol, codeine, and fentanyl on Nav1.5 current in HEK293 cells, and on action potential properties in isolated rabbit ventricular cardiomyocytes. Tramadol reduced Nav1.5 current in a concentration-dependent manner with an IC_{50} of 378.5±33.2 μM. Tramadol caused negative shifts of voltage-gated activation and inactivation, and a delay in recovery from inactivation. In addition, tramadol induced stronger blockade on partially-inactivated channels than on fully-available channels. Tramadol reduced action potential upstroke velocity and action potential amplitude in rabbit cardiomyocytes with a larger amount of reduction at fast pacing rates. Fentanyl and codeine had no effect even when tested at lethal concentrations.

Chapter 5 demonstrates the effects of sulfonylurea antidiabetic drugs. These drugs block the ATP-gated potassium channel (K_{ATP}) which is expressed both in the pancreas and in the heart. We showed that use of these drugs is associated with reduced risk of SCA in patients with diabetes. We hypothesized that these drugs may decrease the risk of SCA by rescuing the shortening of action potential duration which occurs during myocardial ischemia as

a consequence of K_{ATP} channel activation. We measured the effects of glibenclamide, gliclazide, glimepiride, and tolbutamide on simulated ischemia-induced shortening of action potential duration in human induced pluripotent stem cell-derived cardiomyocytes. We found that only glibenclamide significantly rescued this shortening.

NEDERLANDSE SAMENVATTING

Plotse hartstilstanden zijn een wereldwijd gezondheidsprobleem en de belangrijkste oorzaak van sterfte door cardiovasculaire ziekte. Medicijnen, waaronder noncardiale medicijnen (medicijnen die worden gebruikt voor de behandeling van niet-cardiale ziekte), kunnen een invloed hebben op het risico op het ontstaan van een plotse hartstilstand. Dit komt doordat deze medicijnen een effect hebben op de cardiale ion kanalen, die de cardiale actiepotentiaal en de electrische eigenschappen bepalen.

Anti-epileptica worden bestudeerd in **hoofdstukken 2 en 3**. Wij hebben het risico op het ontstaan van een plotse hartstilstand tijdens het gebruik van carbamazepine onderzocht en de mogelijke onderliggende cellulaire electrofysiologische mechanismen in **hoofdstuk 2**. De effecten van carbamazepine (1–100 μM) op actiepotentialen en individuele membraanstromen werden onderzocht in geïsoleerde cardiomyocyten van het konijn en de mens met behulp van de whole-cell patch-clamp techniek. Carbamazepine reduceerde de stijgsnelheid van de actiepotentiaal van cardiomyocyten van konijn en mens zonder andere eigenschappen van de actiepotentiaal te veranderen. Carbamazepine (≥30 μM) reduceerde ook de grootte van de stroom door het cardiale natriumkanaal (Nav1.5), verschoof de voltage-gevoeligheid van inactivatie in een hyperpolariserende richting, en vertraagde het herstel van inactivatie. De grootte van de L-type calcium stroom $I_{Ca,L}$ werd gereduceerd door 100 μM carbamazepine. In **hoofdstuk 3** werden de effecten van vijf andere vaak gebruikte anti-epileptica (gabapentin, lamotrigine, levetiracetam, pregabalin, en valproinezuur), die zijn geassocieerd met een toegenomen risico op het ontstaan van een plotse hartstilstand, onderzocht. In celkweken (HEK293 cellen) met stabiele Nav1.5 expressie werd gevonden dat lamotrigine en valproinezuur de grootte van de stroom door humane Nav1.5 kanalen reduceerde en een verschuiving van steady-state inactivatie naar hyperpolariserende potentialen veroorzaakte, evenals een vertraging in het herstel van inactivatie. De veranderingen in de eigenschappen van Nav1.5 werden weerspiegeld in een reductie van de stijgsnelheid en amplitude van de actiepotentiaal. Gabapentin, levetiracetam en pregabalin hadden geen effect op de Nav1.5 stroom.

In **hoofdstuk 4** werden de effecten van de opiaten tramadol, codeine en fentanyl op Nav1.5 stroom in HEK293 cellen onderzocht, evenals de eigenschappen van actiepotentialen van geïsoleerde ventriculaire cardiomyocyten van het konijn. Tramadol reduceerde de Nav1.5 stroom op een concentratie-afhankelijke manier met een IC_{50} van 378.5±33.2 μM. Tramadol veroorzaakte een negatieve verschuiving van voltage-gevoelige activatie en inactivatie, en een vertraging van herstel van inactivatie. Ook induceerde tramadol een sterkere blokkade van partieel-geïnactiveerde kanalen dan van volledig-beschikbare kanalen. Tramadol reduceerde de stijgsnelheid en amplitude van de actiepotentiaal cardiomyocyten van het konijn. De reductie was groter bij hogere stimulatie frequentie. Fentanyl en codeine hadden geen effect, zelfs niet bij lethale concentraties.

Hoofdstuk 5 toont de effecten van sulfonylureum antidiabetica. Deze medicijnen blokkeren het ATP-gevoelige kalium kanaal (K_{ATP}), dat to expressie komt zowel in de pancreas als in het hart. Wij toonden aan dat gebruik van deze medicijnen is geassocieerd met een kleiner risico op het ontstaan van een plotse hartstilstand in patiënten met diabetes. Wij formuleerden de hypothese dat deze medicijnen dit risico verkleinen doordat zij de verkorting van de duur van de actiepotentiaal, die optreedt tijdens myocardiale ischemie ten gevolge van de activatie van K_{ATP} kanalen. Wij onderzochten de effecten van glibenclamide, gliclazide, glimepiride en tolbutamide tijdens verkorting van de actiepotentiaal duur die ontstond tijdens gesimuleerde ischemie in humane geïnduceerde pluripotente stamcel-gebaseerde cardiomyocyten. Wij vonden dat alleen glibenclamide deze verkorting significant tegenging.

APPENDICES

CONTRIBUTING AUTHORS

In alphabetical order, affiliations at the time this research was conducted.

Gerard J.J. Boink	Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands
Anthonius De Boer	Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands
Marieke T. Blom	Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands
Harsha D. Devalla	Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands
Talip E. Eroglu	Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands
Patrick C. Souverein	Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands
Hanno L. Tan	Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands Netherlands Heart Institute, Utrecht, The Netherlands
Arie O. Verkerk	Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands
Ronald Wilders	Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands

AUTHOR CONTRIBUTIONS

Chapter 2: Carbamazepine increases the risk of sudden cardiac arrest by a reduction of the cardiac sodium current

Lixia Jia, Talip E. Eroglu, Ronald Wilders, Arie O. Verkerk, Hanno L. Tan

H.L.T. conceived and designed the study. T.E.E. structured and carried out the epidemiological studies. A.O.V. structured and designed the patch-clamp studies. L.J. and A.O.V. carried out the patch-clamp experiments. R.W. carried out the statistical analysis of the patch-clamp data. L.J. and TE drafted the first version of the manuscript. All authors contributed to manuscript revision and approved the final version.

Chapter 3: The antiepileptic drugs lamotrigine and valproic acid reduce the cardiac sodium current

Lixia Jia, Arie O. Verkerk, Hanno L. Tan

H.L.T. conceived and designed the study. L.J. and A.O.V. structured and designed the patch-clamp studies. L.J. carried out the patch-clamp experiments, statistical analysis of the patch-clamp data, and drafted the first version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Chapter 4: The opioid tramadol blocks the cardiac sodium channel Nav1.5 in HEK293 cells

Lixia Jia, Arie O. Verkerk, Hanno L. Tan

H.L.T. conceived and designed the study. L.J. and A.O.V. structured and designed the patch-clamp studies. L.J. carried out the patch-clamp experiments and statistical analysis of the patch-clamp data, and drafted the first version of the manuscript. All authors contributed to manuscript revision and approved the final version.

Chapter 5: Sulfonylurea antidiabetics are associated with lower risk of out-of-hospital cardiac arrest: Real-world data from a population-based study

Talip E. Eroglu, Lixia Jia, Marieke T. Blom, Arie O. Verkerk, Harsha D. Devalla, Gerard J.J. Boink, Patrick C. Souverein, Anthonius de Boer, Hanno L. Tan

T.E.E. and H.L.T. conceived the study idea. T.E.E., M.T.B., A.O.V., P.C.S., A.d.B., H.L.T. designed the research. T.E.E. performed the statistical analyses and wrote the manuscript; A.O.V. and L.J. conducted the patch-clamp experiments. P.C.S. worked up the original data to a data matrix ready for statistical analyses. All authors critically revised and approved the manuscript.

LIST OF PUBLICATIONS

1. **Jia L**, Verkerk AO, Tan HL. The opioid tramadol blocks the cardiac sodium channel Nav1.5 in HEK293 cells. (*submitted*)

2. **Jia L**, Verkerk AO, Tan HL. The anti-epileptic drugs lamotrigine and valproic acid reduce the cardiac sodium current. Biomedicines 2023;11(2): 477. DOI: 10.3390/biomedicines11020477

3. **Jia L***, Eroglu TE*, Wilders R, Verkerk AO, Tan HL. Carbamazepine increases the risk of sudden cardiac arrest by a reduction of the cardiac sodium current. Frontiers in Cell and Developmental Biology. 2022;10: 891996. DOI: 10.3389/fcell.2022.891996

4. Eroglu TE*, **Jia L***, Blom MT, Verkerk AO, Devalla HD, Boink GJ, Souverein PC, de Boer A, Tan HL. Sulfonylurea antidiabetics are associated with lower risk of out-of-hospital cardiac arrest: real-world data from a population-based study. British Journal of Clinical Pharmacology. 2021;87:3588-3598. DOI: 10.1111/bcp.14774

*These authors contributed equally to this work

PORTFOLIO

PhD student:	Lixia Jia
PhD period:	September 2018 - May 2023
Supervisors:	Dr. Hanno L. Tan
Co-supervisors:	Dr. Arie O. Verkerk

		ECTS
PhD training Courses		
2021	Scientific Writing	1.5
2021	Writing a Scientific Paper	1.7
2018&2022	Minicourse 'Basics in Electrophysiology'.	0.5
2019	Endnote	0.3
2019	Research Data Management	0.3
2019	Practical Biostatistics	1.5
2019	Data analysis in Matlab	0.7
2019	Laboratory Biosecurity and Biosafety	0.4
Seminars and workshops		
2018-2023	Weekly department meetings	4.0
2018-2023	Weekly progress report	4.0
2018-2021	Weekly Journal Club	3.0
2022	Minicourse 'Basics in Electrophysiology'	0.3
2021	Minicourse 'Basics of GWAS'	0.1
Conferences		
2022	Amsterdam Cardiovascular Sciences, Heart failure and Arrhythmias, Amsterdam (the Netherlands) - *Attendee*	0.3
2022	6th DCVA/NLHI Translational Cardiovascular Research Meeting, Utrecht (The Netherlands) - *Attendee*	0.3
2022	The 46th Annual Meeting of the ESC working group on Cardiac Cellular Electrophysiology, Toledo (Spain) - *Poster presentation*	1.0
2021	12th Rembrandt Symposium (the Netherlands)- *Attendee*	0.2
2019	10th Rembrandt Symposium (the Netherlands) - *Attendee*	0.2

ABOUT THE AUTHOR

Lixia Jia was born on the 28th of September 1991 in Shanxi, China. She started her bachelor's study at Lanzhou University, majoring in Management of Public Health Services. During her undergraduate study, she learned some basic lab knowledge. Throughout her experiences, she found she is more interested in laboratory studies. In 2015, she began to study for a master's degree in hygienic toxicology at Peking University. She obtained her master's degree in Public Health under the supervision of Prof. Weidong Hao in 2018. The same year, she won a four-year scholarship from China Scholarship Council (CSC) and went to the Netherlands for her PhD. She started her PhD study in the group of Dr. Hanno L. Tan, Department of Experimental Cardiology, Academic Medical Center Amsterdam. A major part of her research is the electrophysiological analysis of noncardiac drugs that reduce cardiac excitability and increase the risk of arrhythmias and cardiac arrest. The findings of her PhD research are included in this thesis.

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