Investigation of antiarrhythmic and proarrhythmic mechanisms in transgenic systems

Summary of the PhD thesis

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INTRODUCTION

In public health, one of the major concerns are cardiac arrhythmias. These arrhythmias can occur due to inherited diseases, (LQT, LEOPARD, Andersen-Tawil syndrome) or as a proarrhythmic effect of drugs which can lead to ventricular tachycardia/ventricular fibrillation (VT/VF). "Arrhythmia" refers to any change in the normal sequence and/or shape of electrical impulses during the cardiac cycle. These are very important issues and there is a high need to produce reliable models to study inherited heart diseases, as well as versatile tools for safety pharmacology.

Challenges in the treatment of arrhythmias

Classification of antiarrhythmic drugs (AA) can be done using the Vaughan Williams 4 – level schema or by the mechanistic and clinically relevant Sicilian Gambit. Many of the AA have a complex action on the different ion channels, so they do not necessarily fit to the Vaughan Williams classification (Figure 1). The Sicilian Gambit classifies the agents based on their arrhythmogenic mechanism or action. This type of classification was introduced in 1991.

Class I Drugs that delay fast sodium channel mediated conduction	**Class II** Sympathetic antagonists	**Class III** Drugs that prolong repolarisation	**Class IV** Calcium antagonists
IA Depress phase 0 Delay conduction, Prolong repolarisation Disopyramide, Procainamide, Quinidine **IB** Little effect on phase 0 in normal tissue Depress phase 0 in abnormal tissue Shorten repolarisation or little effect Diphenylhydantoin, Lidocaine, Mexiletine, Tocainide **IC** Markedly depress phase 0 Markedly slow conduction Slight effect on repolarisation Flecainide, Moricizine, Propafenone	Acebutolol Betaxolol Bisoprolol Bucindolol Carvedilol Esmolol Metoprolol Nadolol Propranolol Timolol Others	Amiodarone Azimilide Bretylium Dofetilide Ibutilide Sotalol Tedisamil	Diltiazem Verapamil

Figure 1. The Vaughan Williams classification of antiarrhythmic drugs (adapted from SINGH and WILLIAMS, 1970).

Drug treatment of cardiac arrhythmias is still remains problematic, because of their inadequate effectiveness and a risk of serious complications. The physiological and pathophysiological mechanisms of cardiac arrhythmias are still unclear. Due to this, in many cases the AA have proarrhythmic effects. According to these problems, there is a high need to increase our understanding on the underlying mechanism of those arrhythmias on tissue and cellular level. There is also a high need to develop new, safe and effective antiarrhythmic agents.

Several trials were made to study the possible proarrhythmic effects of the existing antiarrhythmic agents, while there were many cases with induced mortality due to the side effects of the AA. The Cardiac Arrhythmia Suppression Trial (CAST) created to study the effects of the existing AA (Class I/C drugs encainide, flecainide, or moricizine) in patients with asymptomatic, or mildly symptomatic ventricular arrhythmia (six or more ventricular premature beats per hour) after myocardial infarction. The trial was designed to reveal, if the antiarrhythmic agents can reduce the appearance of life threatening arrhythmias in patients with myocardial infarction. After 10 months of follow-up the results showed that lethal cardiac arrhythmias appeared with larger chance in the drug-treated group, compared to the placebo. In conclusion, the use of encainide and flecainide was stopped in the trial, and these drugs were considered to be not safe enough anymore to treat patients with asymptomatic or minimally symptomatic ventricular arrhythmia after myocardial infarction.

The cardiac action potential

In the mammalian heart, the normal pump function is critically depends on proper electrical function. The signal for contraction originates from specialized regions, where pacemaker cells are located. From those pacemaker regions. the trigger propagates through the ventricles. Generation of action potentials (AP) are connected to this myocardial electrical activity of the individual cardiac cell, which can be also detected on surface electrocardiograms. The APs are created by the organized activation and inactivation of ion channels that conduct depolarizing, inward (Na^+ and Ca^{2+}), and repolarizing, outward (K^+), currents. The shape of the APs can differ in various regions of the heart. This can be due to the different expression of the cardiac ion channels, and it makes the propagation of the cardiac signal unidirectional, and it results in a normal cardiac rhythm. In the cardiac

AP five different "phases", can be distinguished. The initial phase (phase 0) is the fast depolarization of the AP. During this phase the fast inward Na^+ channels are activated. The sodium current (I_{Na}) current has a relatively short-lived characteristic (2-3 ms), it creates a large charge influx to membrane depolarization. Beyond activating other currents of the AP (e.g.: Ca^{2+}-current, K^+-currents) this phase is responsible for the rapid impulse propagation. In the phase 1 repolarization, is determined by the transient outward potassium current (I_{to}). The kinetics of this current is similar to the I_{Na}, it is quickly activated and inactivated during depolarization. The magnitude of this phase has important role in shaping the spike-and-dome configuration of the AP. The expression level of I_{to} ion channel subunits can differ across the ventricular wall (and therefore the amplitude of I_{to} as well) the spike-and-dome configuration can be considered a specific "marker" in identifying the ventricular origin of the cell. The phase 2 also called "plateau phase", which is a typical property of the cardiac AP. During this phase the inward L-type calcium current ($I_{Ca,L}$) and outward potassium currents balance each other. They provide a long-lasting isoelectric phase, which has crucial role in cell contraction. $I_{Ca,L}$ is an important player not only in shaping the action potential, but in initiation of intracellular Ca^{2+}-cycle. When $I_{Ca,L}$ slowly inactivates, the outward K^+-currents are still active, and they start the fast late repolarization (phase 3). During the phase 3, the rapid and slow components of delayed rectifiers (I_{Kr} and I_{Ks}) support large outward K^+-current having a mainl role in repolarization. The inward rectifier K^+ current (I_{K1}) has primary role to complete the final phase of the repolarization. This phase 4 represents the resting membrane potential (RMP) during diastole. The RMP of the ventricular cardiomyocytes is determined by I_{K1} and possibly by the Na^+/K^+ pump. In atrial and Purkinje cells, the expression level of I_{K1} is significantly smaller, the resting membrane potential is unstable, and a slow depolarization can be observable (diastolic depolarization), which has important role in the pacemaker function.

Models in cardiovascular pharmacology

To understand better the mechanisms of arrhythmias and heart diseases, there is a high need to develop models, platforms for this purpose. Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) provided a great opportunity to study human heart physiology and disease modelling. However, many studies reported that those hiPSC-CM are immature, and there are major differences in sarcomeric organization and

electrophysiological properties. Studies with patch clamp electrodes consistently reported RMP to be less negative in hiPSC-CM than in adult atrial or ventricular myocardium. This is an alarming finding since correct RMP is mandatory for excitability and refractoriness. One of the possible explanations for a less negative RMP reported in hiPSC-CM, is the absence, or small amplitude of I_{K1}. This current maintains the stable, negative RMP in adult cardiomyocytes (CM). In line with this assumption, current densities of I_{K1} were reported to be low or almost absent in hiPSC-CM cell lines. Generation of Engineered Heart Tissues (EHT) from hiPSC-CM, allows those CM to contract in a coupled manner, and it showed progress in sarcomeric organization and contractile force. EHT format is suitable to use the sharp microelectrode technique to measure action potentials. If EHT format favours maturation in electrophysiological properties, remains unclear.

Generation of transgenic animals, which can express the phenotype of a cardiac disease is also useful tool. In small rodents such as mouse and rat, several diseases were modelled such as LQT, and hypertrophic cardiomyopathy. However, the major problem with those models that their electrophysiological profile shows major differences compared to human, so there is a need to provide transgenic animals which reflecting human heart physiology greater than those small rodents. One of the possible species for this purpose is the rabbit, which is already showed promising results in disease modelling. The electrophysiological profile of the rabbit heart more comparable to human and it is already a commonly used model for cardiovascular pharmacological studies.

The aim of this study is to validate two novel models as possible tools for cardiac safety pharmacology and disease modelling:

1) Compare the I_{K1} current density in hiPSC-CM under two different culture conditions (ML and EHT) and compare them to CM isolated from human right atrial and left ventricular tissue.
2) Investigate whether hiPSC-CM exhibit ventricular, or atrial phenotype based on channel expression (possible expression and function of $I_{K,ACh}$), and specific action potential parameters (repolarization fraction)
3) Study resting membrane potential in hiPSC-CM EHTs using two different techniques (patch clamp and sharp microelectrode technique).

4) Characterise the cellular electrophysiological properties of a novel transgenic LQT5 rabbit model, using the patch clamp technique.

5) Check the *in vivo* electrophysiological parameters of the LQT5 rabbit model and its reaction to arrhythmia provocation.

Methods

Species and preparations

Experiments were carried out in ventricular myocytes enzymatically isolated from rabbit hearts, from human preparations and from hiPSC-CM. The protocols used on rabbit myocytes were approved by the Review Board of the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development, Hungary (22.1/433/003/2010 and XIII./1211/2012) and Ethical Committee for the Protection of Animals in Research at the University of Szeged, Szeged, Hungary (approval number: I-74-9-2009) and conformed to Directive 2010/63/EU of the European Parliament.

Human samples were obtained from heart transplantations and from cardiac surgeries. The experimental protocols used on human samples complied with the Declaration of Helsinki (Cardiovascular Research 1997; 35:2-4). Proper consent was obtained for use of each individual's tissue for experimentation. After explantation, each heart was perfused with cardioplegic solution and kept cold (4 - 6 °C) for 2-4 hours prior to dissection.

Ion current recordings using the patch clamp technique

Transmembrane ion currents were recorded using the whole cell configuration of the patch clamp technique. The tip resistances of the pipettes were between 2-5 MΩ. During the experiments Tyrode solution was used for external solution setted for the measured ion current. The measurements were preformed with the Axopatch 200B Amplifier (Molecular Devices- Axon Instruments, Union-City, USA). The capacity of the cell were measured by a 10 mV depolarizing testpulse, from a holding potential of –90mV. During the recordings the series resistance (4-8 MΩ) was compensated until 50-80 %. Membrane currents were digitally recorded with a 333 Hz software-controlled (Axon pClamp 8.0, 10.0 and ISO2) digitizer (Digidata 1440, Axon Instruments). The current

traces were analyzed using the same softwares (Axon, pClamp 8.0,10.0 and ANA3). The experiments were performed either room, or physiological temperature (37 °C). Contaminating currents were eliminated using pharmacological (selective channel blockers) and electrophysiological (specially designed testpulses).

Current clamp recordings

Action potentials were recorded using the perforated patch (Amphotericin B) configuration of the patch clamp technique. The Axopatch 200 was set to current clamp mode and the experiments performed using Tyrode solution at 37 °C, 1 Hz.

Sharp microelectrode recordings

Sharp microelectrodes were used to record action potentials in right atrial and left ventricular trabeculae and in intact EHT. Microelectrode tip resistances were 20 to 50 MΩ when filled with 3 mM KCl). Action potentials were elicited by field stimulation at 1 Hz: 0.5 ms stimulus, 50% above threshold intensity. The experiments were performed using Tyrode solution at 37 °C.

Quantification of transcript levels

The RNeasy® Plus Mini Kit (Qiagen, Venlo, The Netherlands) was used to isolate total RNA from human heart tissue, cardiomyocytes cultured in a ML and dissociated EHT. For quantification of transcript levels, cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed with the 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) on ABI PRISM 7900HT Sequence detection system (Applied Biosystems, Foster City, California, USA). Relative transcript levels were calculated using the ΔCT-method with glucuronidase beta (Gusb) as a housekeeping gene.

ECG recordings and evaluation

Since different types of anaesthetics can influence cardiac ion channels, rabbits were anaesthetized either with ketamine-S/xylazine or thiopental in the marginal ear-vein. A catheter was inserted into the carotid artery for blood pressure measurement. The right jugular vein was cannulated for i.v. drug administration. Blood pressures and ECGs were continuously recorded, digitized and stored for offline analysis. RR and QT intervals were

measured as the average of 30 beats. Both wild-type and transgenic rabbits received dofetilide (Gedeon Richter Ltd., Budapest, Hungary) 20 $\mu g/kg^{-1}$ over 10 min.

Statistics

All data are expressed as means ± SEM. Statistical analysis was performed with Student's t-test for paired data and One-Way ANOVA. The results were considered statistically significant when p was < 0.05.

RESULTS ANS CONCLUSIONS

Investigation of I_{K1}-current, RMP and action potential parameters in hiPSC-CM

Based on previous publications, the major problem with hiPSC-CM is the low RMP, which can be due to the low expression and small amplitude of the I_{K1}-current. In this study we directly compared the I_{K1} current in hiPSC-CM obtained from the classical monolayer (ML) and from EHT with data recorded from human right atrial (RA) and left ventricular (LV) CM using the whole cell configuration of the patch clamp technique. We checked wether $I_{K,ACh}$ is expressed in hiPSC-CM as a hallmark of atrial phenotype. The EHT format provides an opportunity to record APs using the sharp microelectrode technique, so we compared the RMP values in hiPSC-CM using two different techniques (sharp microelectrode vs. patch clamp technique).

We found Ba^{2+}-sensitive I_{K1} in every hiPSC-CM, and the density of the inward current was similar to, what we measured in human adult CM (RA and LV.) The outward component of the current was significantly larger in LV, than in the other groups. The channel forming subunits of the I_{K1} (Kir 2.1-3) were also larger in LV, compared to ML, EHT and RA, but the neuronal subunit Kir 2.4 was not expressed in any of the groups. Ba^{2+} effectively inhibited the current in all CM. 2 μM carbachol did not influence the current amplitude in hiPSC-CM and LV. The $I_{K,ACh}$ channel forming subunit Kir 3.1 was only expressed in RA. Using sharp microelectrode, the RMP values in EHT were similar to RA and LV. Using patch clamp technique the RMP values were significantly lower in hiPSC-CM than in CM isolated from RA and LV. The action potential duration (APD_{90}) and RMP values showed a large overlap in every group using both techniques. The repolarization fraction was higher in RA, than in LV and EHT, using sharp microelectrode

technique. The results show that the I_{K1} is present in hiPSC-CM, and its inward density is similar to adult CM. The Ba^{2+} effectively inhibited the current even at low concentrations and the absence of the Kir2.4 suggest that the neuronal form of I_{K1} is not expressed in hiPSC-CM. The $I_{K,ACh}$ could not be activated with carbachol, and the Kir3.1 was not expressed in hiPSC-CM, which suggests ventricular phenotype. The measured low RMP in isolated hiPSC-CM using patch clamp technique can be due to technical reasons. The APD_{90} and RMP values showed overlap in every group using both techniques, questioning their reliability to distinguish between atrial and ventricular phenotype in hiPSC-CM. The repolarization fraction was higher in RA, than in EHT and LV using sharp microelectrodes, which suggest that it can be a useful tool to discriminate between atrial and ventricular phenotype in hiPSC-CM.

In vitro and *in vivo* investigation of a novel, transgenic LQT5-syndrome rabbit model

The reliable assessment of proarrhythmic risk of compounds under development, remains an elusive goal. Current safety guidelines are focusing on the effects of blocking the KCNH2/HERG ion channel in tissues and animals with intact repolarization. Novel models with better predictive value are needed that more closely reflect the conditions in patients with cardiac remodelling and reduced repolarization reserve.

We have developed a model for the long QT syndrome type-5 inrabbits (LQT5) with cardiac-specific overexpression of a mutant (G52R) KCNE1 β-subunit of the channel that carries the slow delayed-rectifier K^+-current (I_{Ks}). ECG parameters, including short-term variability of the QT interval (STVQT), a biomarker for proarrhythmic risk, and arrhythmia development were recorded. *In vivo*, arrhythmia susceptibility was evaluated by i.v. administration of the I_{Kr} blocker dofetilide. K^+-currents weremeasured with the patch clamp technique. Patch clamp studies in ventricular myocytes isolated from LQT5 rabbits revealed accelerated I_{Ks} and I_{Kr} deactivation kinetics. The density of the I_{to} was larger in transgenic animals, while the density of the inward and the outward I_{K1} current was similar in both groups. At baseline, LQT5 animals exhibited slightly, but significantly prolonged heart-rate corrected QT index (QTi) and increased STVQT. Dofetilide provoked Torsade-de-Pointes arrhythmia in a greater proportion of LQT5 rabbits, paralleled by a further increase in STVQT. We have created a novel transgenic LQT5 rabbit model with increased susceptibility to drug-induced arrhythmias that may represent a useful model for testing proarrhythmic potential and for investigations of the

mechanisms underlying arrhythmias and sudden cardiac death due to repolarization disturbances.

SUMMARY AND POTENTIAL SIGNIFICANCE

The conclusions and main findings of the present thesis are as follows:

1. HiPSC-CM can possess robust I_{K1} current densities, which in ML, reached values of human LV-CM and, in EHT, that of RA-CM under identical experimental conditions.
2. Technical issues related to patch clamping of small cells probably contribute to the reported low RMP in hiPSC-CM. HiPSC-CM exhibit features of both an atrial (I_{K1} and RMP) and ventricular phenotype (absence of $I_{K,ACh}$ and low repolarization fraction). Low I_{K1} and depolarized RMP are not inherent characteristics of hiPSC-CM.
3. A novel transgenic LQT5 rabbit model based on the cardiac-specific overexpression of human KCNE1 carrying a G52R missense was successfully created. These rabbits exhibit reduced repolarization reserve, but no striking repolarization disturbances or serious ventricular arrhythmias at baseline. However, LQT5 rabbits are more susceptible to arrhythmias than wild-type littermates upon repolarization stress, indicating that they may be suitable to model the challenging clinical situation of 'silent' LQT.
4. This model can also provide further insights into the mechanisms underlying arrhythmias and sudden cardiac death based on repolarization disturbances and may represent a novel model for testing the proarrhythmic potential of new drugs under development.
5. We can conclude that due to their large similarity to human heart both models can be reliable tools in future for cardiovascular pharmacology in *in vitro* and *in vivo* disease modelling studies.

LIST OF PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

Full length papers

I. **Horváth A**, Lemoine MD, Loser A, Mannhardt I, Flenner F, Uzun AU, Neuber C, Breckwoldt K, Hansen A, Girdauskas E, Reichenspurner H, Willems S, Jost N, Wettwer

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I. **Horváth A**, Uzun A, Voller I, Breckwoldt K, Neuber C, Ansen A, Varró A, Eschenhagen T, Christ T, Mesterséges izomszövetekből izolált pluripotens őssejtekből származtatott szívizomsejtek elektrofiziológiai tulajdonságai (Electrophysiological properties of human induced pluripotent stem cell derived cardiomyocytes isolated from engineeredheart tissues) CARDIOLOGIA HUNGARICA 45:(Suppl. D) pp. D31-D32. (2015)

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III. **Horváth A**, Gurr K, Ismaili D, Mannhardt I, Ulmer B, Hansen A, Eschenhagen T, Christ T, Properties of the sodium-calcium exchanger and the Na^+/K^+-ATPase in human induced pluripotent stem cell-derived cardiomyocytes. EP EUR.;19: iii7-iii7. (2017)

IV. **Horváth A**, Gurr K, Ismaili D, Mannhardt I, Ulmer B, Hansen A, Jost N, Eschenhagen T, Christ T, A nátrium-kalcium cseremechanizmus és a Na^+/K^+-ATPáz vizsgálata humán indukált pluripotens őssejtekből származtatott szívizomsejtekben (Investigation of the sodium-calcium exchanger and the Na^+/K^+ -ATPase in human induced pluripotent stem cell-derived cardiomyocytes). CARDIOLOGIA HUNGARICA 47:(Suppl.C) p. C48. (2017)

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