In vitro study of the electrophysiological properties of several cardioactive drugs in mammalian hearts

PhD Thesis

Zsolt A. Nagy, *MSc*

Department of Pharmacology & Pharmacotherapy
Faculty of Medicine
Albert Szent-Györgyi Medical and Pharmaceutical Center
University of Szeged
Szeged, Hungary

2008

Contents

List of publications related to the subject of the Thesis 4

1. Introduction 5

2. Major specific experimental goals 10

3. Methods 11
3.1. Experiments in animals 11
3.1.1 Preparations 11
3.1.2 Conventional microelectrode technique 11
3.1.3. Whole cell patch-clamp measurements 12
3.2. Experiments in human cardiac preparations 16
3.2.1 Human tissue preparation 16
3.2.2. Experimental techniques: action potential and whole cell patch-clamp measurements 16
3.3. Drugs 16
3.3. Statistical analysis 17

4. Results 18
4.1. The effect of SEA-0400, a newly developed NCX inhibitor devoid of I_{Ca} blocking property, on the NCX and I_{Ca} currents in dog ventricular preparations and Purkinje fibres 18
4.1.1 Effect of SEA-0400 on early and delayed afterdepolarizations 18
4.1.2 Effect of SEA-0400 on L-type Ca^{2+} current and Ca^{2+} transients 19
4.2. Electrophysiological effects of series of molecules that combine the hydroxy-benzopyran ring of vitamin E with the methylsulfonyl-aminophenyl moiety of class III antiarrhythmic drugs 20
4.3. In vitro electrophysiological effects of SZV-123, a new amiodarone-like (combined Class I/B+III) antiarrhythmic drug, on the action potential parameters and main repolarizing transmembrane currents in dog and rabbit cardiac preparations 23
4.4. The role of slow delayed rectifier potassium current in human ventricular muscle 27
4.4.1 Effect of I_{Ks} and I_{Kr} blockade on normal human ventricular muscle action potential duration in the absence of sympathetic stimulation 27
4.4.2 The effect of I_{Ks} block during increased sympathetic activation following attenuation of repolarization reserve 29
4.5. Contribution and relative role of the rapid delayed and inward rectifier potassium channels (I_{Kr} and I_{K1}) in human, dog and rabbit ventricular repolarization 30

5. Discussion 33
5.1. The electrophysiological effects of SEA-0400, a newly developed NCX inhibitor devoid of I_{Ca} blocking property, on the NCX and I_{Ca} currents in dog cardiac preparations 33
5.2. Electrophysiological effects of series of molecules that combine the hydroxy-benzopyran ring of vitamin E with the methylsulfonyl-aminophenyl moiety of class III antiarrhythmic drugs 35
5.3. In vitro electrophysiological effects of SZV-123, a new amiodarone-like antiarrhythmic drug, on the action potential parameters and main repolarizing transmembrane currents in dog and rabbit cardiac preparations 36

5.4. The role of slow delayed rectifier potassium current (I_{Ks}) in human ventricular muscle 36
5.5. Contribution and relative role of the rapid delayed and inward rectifier potassium channels (I_{Kr} and I_{K1}) in human, dog and rabbit ventricular repolarization 39

6. Summary: conclusions and potential significance 42

7. References 44

8. Acknowledgements 50

9. Annex. Publications related to the subject of the Thesis..... 51

List of publications related to the subject of the PhD Thesis

Full length papers

I. Zsolt A. Nagy, László Virág, András Tóth, Péter Biliczki, Károly Acsai, Tamás Bányász, Péter Nánási, Julius Gy. Papp, András Varró. Selective inhibition of sodium-calcium exchanger by SEA-0400 decreases early and delayed afterdepolarization in canine heart.
British Journal of Pharmacology 2004; 143, 827−831
IF(2004)= 3.825 Nr. citations: 6

II. Norbert Jost, László Virág, Miklós Bitay, János Takács, Csaba Lengyel, Péter Biliczki, **Zsolt A. Nagy**, Gábor Bogáts, David A. Lathrop, Julius G. Papp, András Varró. Restricting excessive cardiac action potential and QT prolongation.
Circulation 2005; 112: 1392-1399
IF(2005): 10.94 Nr. citations: 39

III. Maria Koufaki, Christina Kiziridi, Panagiota Papazafiri, Athanasios Vassilopoulos, András Varró, **Zsolt A. Nagy**, Attila Farkas and Alexandros Makriyannis. Synthesis and biological evaluation of benzopyran analogues bearing class III antiarrhythmic pharmacophores.
Bioorganic & Medicinal Chemistry 14, 6666-6678, 2006.
IF(2006) = 2.624 Nr. citations: 3

Quotable abstracts

IV. Norbert Jost, László Virág, Viktória Szűts, Csaba Lengyel, Péter Biliczki, **Zsolt A. Nagy**, György Seprényi, Péter P. Kovács, János Szabad, Julius Gy. Papp, András Varró. Comparison of the contributing effect of the inward rectifier potassium current to repolarization in human, dog and rabbit hearts
Cardiologia Hungarica, 36, SupplA, A21, 2006.

1. Introduction

Cardiovascular diseases, and in particular, cardiac arrhythmias, such as ventricular fibrillation have a leading role in mortality in developed countries. The most serious ventricular arrhythmia – ventricular fibrillation – causes the death of more than 3.000.000 people all over the world and 300.000 – 350.000 people in the USA and Europe annually, which statistically means that one person dies every minute on each continent. In Hungary exact data are not available, but according to calculations there are 25.000 – 26.000 sudden cardiac death cases annually, or 50 – 60 deaths per day. In the majority of the cases sudden cardiac death occurs when victims are not in hospital, consequently, survival probability is very low. Most frequently (50 %) the background of the on-the-spot diagnosed circulation collapse is ventricular tachycardia /fibrillation/. Sudden cardiac death is often the very first sign of the symptom-free cardiovascular disease. Sudden cardiac death is a complex national health problem affecting families and having significant social and economic consequences, since usually it is the head of the family, a seemingly healthy man, who dies tragically. Survivors of the crisis can live a life of full volume in good conditions provided that they get the most appropriate treatment. Accordingly, cardiac arrhythmias represent a major area of cardiovascular research [1, 2]. One of the main goals of pharmacological research is to develop a safe ventricular antiarrhythmic drug that can be applied either in acute cases or for treating postinfarction patients.

According to the classification of Vaughan Williams (1970), based on electrophysiological actions, the antiarrhythmic drugs can be defined by four classes [3-5]. Class I consist of antiarrhythmic agents that block sodium channels, reducing the maximum increase rate of depolarization (V_{max}). Class II agents are the β-blockers, the Class III drugs act through delaying repolarization of cardiac myocytes and thus cause a lengthening of APD (potassium-channel blockers), while Class IV antiarrhythmics block calcium currents in cardiac tissue.

Concepts regarding the treatment of cardiac arrhythmias changed significantly in the past decade, owing to the revolutionary development of electrophysiological methods (patch-clamp, molecular biology). The Cardiac Arrhythmia Suppression Trial (CAST) showed that flecainide and encainide, two Class I/C sodium channel blocker antiarrhythmic drugs, increased mortality rates approximately threefold compared with placebo due to proarrhythmic effects [6]. Consequently, the interest of drug development for treatment of

ventricular tachycardia (VT) and atrial fibrillation (AF) has been shifted toward those agents that prevent and terminate re-entrant arrhythmias by prolonging the action potential duration (APD) and effective refractory period (ERP), resulting in an increase in arrhythmia wavelength and a block development within the re-entrant circuit.

Class III antiarrhythmic action, *i.e.* lengthening of cardiac action potential duration (APD) and prolongation of the repolarization, is usually caused by blockade of one or more potassium channels [3]. Sodium channels are not affected, thus conduction velocity remains unchanged. A great number of non-cardiac drugs cause lengthening of repolarization in both ventricular muscle cells and Purkinje fibres by using a similar mode of action [7-11].

One arrhythmogen factor that can result in ventricular arrhythmias occurring in myocardial ischaemia or poisoning with digitalis is delayed afterdepolarization (DAD), which arises in heart muscle cells following Ca^{2+} overload. Reducing the incidence of these trigger mechanism (DAD) or their pharmacological blockade would be extremely desirable from a clinical point of view.

Maintenance of the Ca^{2+} homeostasis in the myocardium is mainly regulated by the sodium-calcium exchanger (NCX) (12,13). Mammalian Na^+/Ca^{2+} exchangers are members of three branches of a much larger family of transport proteins [the CaCA (Ca^{2+}/cation antiporter) superfamily] whose main role is to provide control of Ca^{2+} flux across the plasma membranes or intracellular compartments. Since cytosolic levels of Ca^{2+} are much lower than those found extracellularly or in sequestered stores, the major function of NCX is to extrude Ca^{2+} from the cytoplasm. The exchangers are, however, fully reversible and thus, under special conditions of subcellular localization and compartmentalized ion gradients, NCX may allow Ca^{2+} entry and may play more specialized roles in Ca^{2+} movement between compartments. The NCX branch of Na^+/Ca^{2+} exchangers comprises three members: NCX1 has been most extensively studied, and is broadly expressed with particular abundance in heart, brain and kidney, NCX2 is expressed in brain, and NCX3 is expressed in brain and skeletal muscle [14].

It is known that NCX, at the forward mode, extrudes Ca^{2+} from the cell to the extracellular space during diastole, at relatively low free cytoplasmic Ca^{2+} concentration and negative transmembrane potential. Since the extrusion of one Ca^{2+} is coupled with 3 Na^+ entering the cell, during the forward mode of the NCX net inward current is carried, which can cause substantial depolarization leading to early (EAD) and delayed (DAD) after-depolarizations, especially when intracellular Ca^{2+} is elevated. EAD and DAD is generally thought to play an important role in arrhythmogenesis [1, 15, 16], especially in conditions

where potassium conductance is decreased, such as heart failure [2]. Therefore one may speculate that specific blockers of NCX could be potential antiarrhythmics in dysrhythmias related to Ca^{2+} overload [16, 17]. This hypothesis could not be directly tested since the available NCX inhibitors, at least in higher concentrations, also decreased the L-type calcium current (I_{Ca}) which in turn decreased intracellular Ca^{2+} load, thereby indirectly changing the magnitude of NCX. In 1996 it was found that KB-R7943 (2-[2-[4-94-nitrobenzyloxy)phenyl]ethyl]isothiourea), an effective inhibitor of NCX in the reverse mode but not in the forward mode [18], reduced the incidence of ischaemia and reperfusion arrhythmia induced by calcium overload [19, 20]. However, KB-R7943 also inhibits the L-type calcium current [21] which makes the interpretation of its antiarrhythmic effect rather uncertain.

In 2001, Matsuda et al. reported SEA-0400 (2-[4-[(2,5-difluorophenyl)methoxy]phe-noxy]-5-ethoxy-aniline), a newly developed, more potent, and selective NCX inhibitor [22]. It is completely coincidental that SEA-0400 and KB-R7943 have a common benzyloxyphenyl structure, suggesting that this portion is important for inhibitory action against NCX [21]. SEA-0400 is highly specific for NCX because it hardly inhibits other receptors, channels and transporters. In 2002, SN-6 was found by screening newly synthesized benzyloxyphenyl derivatives for NCX1 inhibition [23]. This compound showed inhibitory potency for NCX1 similar to KB-R7943, but was more specific for NCX1 than KB-R7943 [24].

One of the goals of my PhD project was to investigate the effect of SEA-0400 on the NCX and I_{Ca} currents of dog ventricular myocytes, and also on the formation of EAD and DAD in the dog ventricular muscle and Purkinje fibres, using the conventional microelectrode and patch-clamp techniques.

In the case of antiarrhythmic drugs the delicate balance between drug efficacy and unexpected adverse side effects is narrower than in any other class of therapeutic agents. Sotalol, amiodarone, ibutilide and dofetilide are moderately effective in patients with chronic atrial fibrillation. However, amiodarone appears to be most efficacious [25]. Moreover, amiodarone and dofetilide is efficacious in patients who have had a myocardial infarction and those with heart failure [26]. The safety of commercially available d,l-sotalol in these patients is poorly understood. *Torsades de pointes* is the most serious adverse effect of sotalol [27] and dofetilide [28]. Amiodarone has minimal proarrhythmic risk, but has numerous noncardiac toxicities that require frequent monitoring[29, 30]. Dronedarone, a noniodinated benzofuran derivative, has been shown to be more effective in vivo than amiodarone in several arrhythmia models, particularly in preventing ischemia- and reperfusion-induced

ventricular fibrillation and in reducing mortality [31]. However, further experimental studies and long-term clinical trials are required to provide additional evidence of efficacy and safety of this drug [31]. Azimilide statistically reduced the incidence of new atrial fibrillation in recent survivors of myocardial infarction at high risk for sudden cardiac death [32]. In addition, class III antiarrhythmic agents are increasingly being used as adjunct therapy to decrease the frequency of ICD discharges in patients with ventricular arrhythmias and implantable cardioverter defibrillators (ICDs) [33]. The antiarrhythmic efficacy of most pure class III drugs is compromised by their inherent property to induce excessive lengthening of the action potential (reverse frequency dependence) and their inability to prolong the action potential when most needed, namely during tachycardia [34]. Overall, an ideal antiarrhythmic agent does not exist, and drug selection should be highly individualized [35, 36].

Based on these results in antiarrhythmic field we synthesized a series of molecules that combine several modes of actions to find a drug that has powerful antiarrhythmic potential with lack of proarrhythmic side effects [37]. One possible direction of development was to test the combination of the hydroxy-benzopyran ring of vitamin E with the methylsulfonyl-aminophenyl moiety of class III antiarrhythmic drugs. Specifically, the new compounds combine pharmacophores identified for the most active Class III antiarrhythmics. Thus, they contain two aromatic rings, one methylsulfonyl amino group, and at least one tertiary amine, such as a 1,4-piperazine or methylamine moiety.

Evaluation of the antiarrhythmic and antioxidant activity of the new compounds was carried out on isolated rat heart preparations using the non-recirculating Langendorff mode. The new analogues were present, at 10 μM concentration, during ischaemia and reperfusion. Selected compounds were further studied by a conventional microelectrode method in order to get insight into their cellular mode of action.

Another possible area for development of new antiarrhythmics are amiodarone-like drugs, that combine Class IB+III antiarrhythmic effects. Our chemical collaboration partner designed and developed a new agent, SZV-123, that based on some preliminary screening proved to have strong antiarrhythmic potential, therefore it was selected for a more intensive electrophysiological screening project. We have analysed the effects of SZV-123 on the action potential parameters and main repolarizing transmembrane ionic currents by applying the conventional and whole-cell patch-clamp techniques.

Ventricular repolarization is governed by a fine balance between inward currents, such as the fast sodium (I_{Na}) and the L-type calcium (I_{Ca}) currents, and outward currents, such as the transient outward (I_{to}), rapid delayed rectifier (I_{Kr}), slow delayed rectifier (I_{Ks}) and inward

rectifier (I_{K1}) potassium currents [38]. Under normal conditions impairment or block of one type of outward potassium currents can not be expected to cause excessive and potentially dangerous APD lengthening [39, 40], since other potassium currents may provide sufficient repolarizing capacity, which can be considered as a "repolarization reserve" [41]. However, in situations where the density of one or more types of potassium channels is decreased by inheritance or remodelling [42, 43], inhibition of other potassium channels may lead to unexpectedly augmented APD prolongation, resulting in proarrhythmic reactions. Genetic channelophathies of certain potassium channels, which normally contribute to repolarization, can attenuate the capability of the heart to repolarize.

The rapid component of the delayed rectifier potassium current (I_{Kr}) has been identified in several mammalian species, including human [44-47]. Pharmacological agents that selectively block I_{Kr} (e.g., E-4031, sotalol and dofetilide) markedly increase APD, QT duration and ventricular refractoriness, and high doses of these drugs are associated with the induction of *Torsades de Pointes* [48, 49]. Mutations in ion channel genes, including HERG and KCNE2, that suppress I_{Kr} result in a specific form of the inherited long QT syndrome, the LQT2, which is also associated with rhythm disorders and an increased incidence of sudden cardiac death [50]. As such, I_{Kr} plays a major role in action potential repolarization in health and in specific cases of arrhythmogenesis [34, 39, 44, 45].

The role of the slow delayed rectifier potassium current (I_{Ks}) in human ventricular muscle action potential repolarization, on the other hand, has been often debated. As with I_{Kr}, I_{Ks} has been identified in several mammalian species, including humans [44-46, 51, 52] and mutations in KCNQ1 and KCNE1, the alpha and beta-subunits of the I_{Ks} potassium channel, are associated with another specific form of the inherited long QT syndrome, LQT1 [50, 53]. We previously reported that complete pharmacological block of I_{Ks}, by either chromanol 293B or L-735,821, has little effect on APD in isolated dog and rabbit ventricular muscle over a wide range of physiologic pacing frequencies [39, 54]. These findings led us to speculate that I_{Ks} normally plays little role in ventricular muscle action potential repolarization. However, when APD is abnormally long, I_{Ks} likely provides an important safety mechanism that when removed increases arrhythmic risk [39, 40]. Our previously reported findings have now been confirmed by other investigators [55, 56] and supported by computer simulations suggesting that I_{Ks} does not play a role in adaptations of APD to changes in heart rate [57]. However, the role of I_{Ks} in human ventricular muscle remains controversial; although, our preliminary characterization of I_{Kr} [47] and I_{Ks} [52] in isolated human ventricular myocytes suggests that these currents behave much the same as they do in isolated dog [39, 45] and

rabbit [46, 54] ventricular myocytes. The purpose of the present study, therefore, was to confirm our initial findings while further elucidating the role of I_{Ks} in normal human ventricular muscle action potential repolarization and in preparations where repolarization reserve was attenuated and sympathetic activation was increased by exogenous dofetilide and adrenaline.

In addition, we investigated and compared the role and relative contribution of two particularly important repolarizing potassium currents I_{Kr} and I_{K1} to the repolarization in human, dog and rabbit hearts at the cellular levels.

2. Major specific experimental goals

a.) To study the effect of SEA0400, a newly developed NCX inhibitor devoid of I_{Ca} blocking property, on the NCX and I_{Ca} currents of dog ventricular myocytes, and also on the formation of EAD and DAD in the dog ventricular muscle and Purkinje fibres.

b.) To investigate the *in vitro* electrophysiological effects of a series of molecules that combine the hydroxy-benzopyran ring of vitamin E with the methylsulfonyl-aminophenyl moiety of Class III antiarrhythmic drugs, that can represent novel cardioprotective compounds with improved efficacy in the treatment of life-threatening arrhythmias in rabbit cardiac preparations.

c.) To investigate the *in vitro* electrophysiological effects of SZV-123, a new amiodarone-like (combined Class IB+III) antiarrhythmic, that can be a novel cardioprotective compound with improved efficacy in the treatment of life-threatening arrhythmias, in dog and rabbit cardiac preparations.

d.) To elucidate the role of I_{Ks} current in normal human ventricular muscle, and in preparations where repolarization reserve was attenuated and sympathetic activation was increased by exogenous dofetilide and adrenaline.

e.) To study and compare the role and relative contribution of rapid delayed rectifier I_{Kr} and inward rectifier potassium currents (I_{K1}) in the repolarization of human, dog and rabbit ventricular muscle.

3. Methods

Experiments were carried out in ventricular preparations isolated from dog, rabbit hearts and from undiseased human cardiac ventricular preparations.

3.1. Experiments in animals

Untreated New-Zealand white rabbits and adult mongrel dogs of either sex (body weights 1.5-2 kg and 8-20 kg, respectively) were used for the study. All experiments were conducted in compliance with the *Guide for the Care and Use of Laboratory Animals* (USA NIH publication No 85-23, revised 1985). The protocols were approved by the review board of Committee on Animal Research (CAR) of the Albert Szent-Györgyi Medical University (54/1999 OEj).

3.1.1. Preparations

Endocardial preparations (obtained from papillary and trabeculae muscles) were isolated from the right ventricle of hearts removed from anaesthetized (sodium pentobarbital, 30 mg/kg iv.) mongrel dogs of either sex. Free running false tendons of Purkinje fibres were excised from the right or the left ventricle of the hearts. The preparations were placed in a tissue bath and allowed to equilibrate for at least 2 hours while superfused with oxygenated (95 % O_2 – 5 % CO_2) Locke's solution (flow 4-5 ml/min) warmed to 37 °C (pH 7.35 ± 0.05) and containing (in mM) NaCl 123, KCl 4.7, $NaHCO_3$ 20, $CaCl_2$ 1.8, $MgCl_2$ 1.0 and D-glucose 10. Preparations were oxygenated also in the tissue bath directly.

3.1.2. Conventional microelectrode technique

Untreated New-Zealand white rabbits and adult mongrel dogs of either sex were used. Following anaesthesia (sodium pentobarbital, 30 mg/kg administered intravenously), the heart of each animal was rapidly removed through right lateral thoracotomy. The hearts were immediately rinsed in oxygenated Locke's solution containing (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 20, $CaCl_2$ 1.8, $MgCl_2$ 1.0 and D-glucose 10. The pH of this solution was 7.35 ± 0.05 when gassed with 95% O_2 and 5% CO_2 at 37 °C. The tip of the papillary muscles obtained from the right ventricle were individually mounted in a tissue chamber (volume 50 ml). Each ventricular preparation was initially stimulated (HSE (Hugo Sachs Elektronik) stimulator type 215/II, March-Hugstetten, Germany) at a basic cycle length (BCL) of 1000 ms (frequency=1

Hz), using 2 ms rectangular constant voltage pulses isolated from ground and delivered across bipolar platinum electrodes in contact with the preparation. Each preparation was allowed to equilibrate for least 1, while they were continuously superfused with Tyrode's solution. Temperature of the superfusate was kept constant at 37 °C. Transmembrane potentials were recorded using conventional microelectrode techniques. Microelectrodes filled with 3M KCl and having tip resistances of 15-20 Mohm were connected to the input of a high impedance electrometer (HSE microelectrode amplifier type 309), which was connected to ground. The first derivative of transmembrane potentials was electronically obtained by an HSE differentiator (type 309). The voltage outputs from all amplifiers were displayed on a dual beam memory oscilloscope (Tektronix 2230 100 MHz digital storage oscilloscope, Beaverton, OR, USA).

The maximum diastolic potential, action potential amplitude and action potential duration (APD) measured at 50 and 90% repolarization ($APD_{50\text{-}90}$) were obtained using a software developed in our department (HSE-APES) on an IBM 386 microprocessor based personal computer connected to the digital output of the oscilloscope. After control measurements the preparations were superfused for 60 min with Tyrode's solution containing the compound under study, and then the electrophysiological measurements were resumed.

3.1.3. Whole cell patch-clamp measurements

Isolation of rabbit myocytes

Single ventricular myocytes were obtained by enzymatic dissociation. The animals were sacrificed by cervical dislocation after receiving 400 IU/kg heparin intravenously. The chest was opened and the heart was quickly removed and placed into cold (4°C) solution with the following composition (mM): NaCl 135, KCl 4.0, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 10, $NaHCO_3$ 4.4, Glucose 10, $CaCl_2$ 1.8, (pH 7.2). The heart was mounted on a modified, 60 cm high Langendorff column and perfused with oxygenated and pre-warmed (37 °C) solution mentioned above. After washing out blood (3-5 min) it was perfused with nominally Ca-free solution until the heart ceased (approx. 3-4 minutes). The digestion was performed by perfusion with the same solution supplemented with 0.33 mg/ml (90 U/ml) Collagenase (SIGMA Chemical, St. Louis, MO, USA, Type I) and 0.02 mg/ml Pronase E (SIGMA) with 0.1% Albumin using a perfusion pump (flow rate approx. 15 ml/min). In the 15^{th} minute of the enzyme perfusion the calcium concentration was elevated by 200 µM. After 30-35 minutes the heart was removed from the cannula and was placed into enzyme free solution containing 1.8 mM $CaCl_2$ and 1% Albumin and was equilibrated at 37°C for 10 minutes.

Then the tissue was cut into small fragments. After gentle agitation, the cells were separated from the chunks by filtering through nylon mesh. Sedimentation was used for harvesting cells; as soon as most myocytes reached the bottom of the vessel the supernatant was removed and replaced by HEPES buffered Tyrode solution containing (mM): NaCl 144, NaH_2PO_4 0.33, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 0.53, Glucose 5.5, and HEPES 5.0 at pH of 7.4 (adjusted with NaOH). This procedure was repeated twices. The cells were stored at room temperature in HEPES buffered Tyrode solution.

Isolation of dog myocytes

Ventricular myocytes were enzymatically dissociated from hearts, removed from mongrel dogs of either sex weighing 10-20 kg following anaesthesia (sodium pentobarbital, 30 mg/kg iv.). The hearts were immediately placed in cold (4 °C) normal Tyrode solution. A portion of the left ventricular wall containing an arterial branch large enough to cannulate was then perfused in a modified Langendorff apparatus at a pressure of 60 cmH_2O with solutions in the following sequence: (1) normal Tyrode solution (10 min), (2) Ca^{2+}-free solution (10 min), and (3) Ca^{2+}-free solution containing collagenase (type I, 0.66 mg/ml, Sigma) and bovine serum albumin (fraction V, fatty acid free, 2 mg/ml, Sigma) (15 min). Protease (type XIV, 0.12 mg/ml, Sigma) was added to the final perfusate and another 15-30 min of digestion was allowed. Portions of the left ventricular wall judged to be well digested were diced into small pieces and placed either in Kraft-Brühe (KB) solution or in Ca^{2+}-free solution supplemented with $CaCl_2$ (1.25 mM) for 15 min. Next, these tissue samples were gently agitated in a small beaker to dislodge single myocytes from the extracellular matrix. All cell suspensions resulting from this dissociation procedure contained a mixture of subepicardial, midmyocardial and subendocardial myocytes. During the entire isolation procedure, solutions were gassed with 100% O_2 while their temperatures were maintained at 37 °C. Myocytes were allowed to settle to the bottom of the beaker for 10 min, and then the supernatant was replaced with fresh solution. This procedure was repeated three times. Myocytes placed in KB solution were stored at 4 °C; those placed in Tyrode solution were maintained at 12-14 °C prior to experimentation. Cells that were stored in KB solution or immediately placed in 1.25 mM calcium containing solution had the same appearance and there were no discernible differences in their characteristics.

Compositions of solutions used for cell isolation**.** i) Normal Tyrode solution (mM): NaCl 144, NaH_2PO_4 0.33, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 0.53, Glucose 5.5, and HEPES 5.0 at pH of 7.4. (adjusted with NaOH). ii) Ca^{2+}-free solution (mM): NaCl 135, KCl 4.7, KH_2PO_4

1·2, $MgSO_4$ 1.2, HEPES 10, $NaHCO_3$ 4.4, glucose 10 and taurine 20 (pH 7.2 adjusted with NaOH). iii) KB solution (mM): KOH 90, L-glutamic acid 70, taurine 15, KCl 30, KH_2PO_4 10, $MgCl_2$ 0.5, Hepes 10, glucose 11 and EGTA 0.5 (pH 7.3 adjusted with KOH).

Experimental protocols for potassium current measurements

One drop of cell suspension was placed within a transparent recording chamber mounted on the stage of an inverted microscope (TMS, Nikon, Tokyo, Japan), and individual myocytes were allowed to settle and adhere to the chamber bottom for at least 5 min before superfusion was initiated. Only rod shaped cells with clear cross striations were used. HEPES buffered Tyrode's solution (composition see above) served as the normal superfusate. Patch-clamp micropipettes were fabricated from borosilicate glass capillaries (Clark, Reading, UK) using a P-97 Flaming/Brown micropipette puller (Sutter Co, Novato, CA, USA). These electrodes had resistances between 1.5 and 2.5 MΩ when filled with pipette solution containing (in mM): K-aspartate 100, KCl 45, ATP 3, $MgCl_2$ 1, EGTA 10 and HEPES 5. The pH of this solution was adjusted to 7.2 by KOH. Cell capacitance was measured by applying a 10 mV hyperpolarizing pulse from -10 mV, while the holding potential was -90 mV. The capacity was measured by integration of the capacitive transient divided by the amplitude of the voltage step (10 mV).

When measuring K^+ currents, nisoldipine (1 mM) (gift from Bayer AG, Leverkusen, Germany) was added to the external solution to eliminate inward L-type Ca^{2+} current (I_{Ca}). The rapid I_{Kr} and slow I_{Ks} components of the delayed rectifier potassium current were separated by using the selective I_{Kr} blocker E-4031 (1 mM, Institute for Drug Research, Budapest, Hungary) or the I_{Ks} blockers L-735,821 (100 nM, a gift from Merck-Sharpe & Dohme, West-Point, PA, USA), chromanol 293B (Aventis Pharma, Frankfurt, Germany) or HMR 1556 (1 μM, Aventis Pharma). Membrane currents were recorded with Axopatch-1D and 200B patch-clamp amplifiers (Axon Instruments, Union City, CA, USA.) using the whole-cell configuration of the patch-clamp technique. After establishing a high (1-10 Gohm) resistance seal by gentle suction, the cell membrane beneath the tip of the electrode was disrupted by suction or by application of 1.5 V electrical pulses for 1-5 ms. The series resistance was typically 4-8 Mohm before compensation (50-80%, depending on the voltage protocols). Experiments where the series resistance was high, or substantially increased during measurement, were discarded. Membrane currents were digitized using a 333 kHz analogue-to-digital converter (Digidata 1200 and 1322A, Axon Instruments) under software control (pClamp 7.0 and 8.0 Axon Instruments). Analyses were performed using Axon

(pClamp 8.0) software after low-pass filtering at 1 kHz. All patch-clamp data were collected at 37 ˚C.

Experimental protocols for calcium current measurements

The L-type calcium current (I_{Ca}) was recorded in three dogs by the whole-cell configuration of the patch-clamp technique in HEPES buffered Tyrode solution supplemented with 3 mM 4-aminopyridine in order to block transient outward current. A special solution was used for filling the micropipettes (composition in mM: KCl 110, KOH 40, EGTA 10, HEPES 10, TEACl 20, MgATP 5, GTP 0.25, the pH was adjusted to 7.2 with KOH). All experiments were carried out at 37^{o} C.

Experimental protocols for calcium-transient measurements

Changes in intracellular free calcium concentration ([Ca^{2+}]) were assessed during repetitive contraction–relaxation cycles ("calcium transients") by the "ratiometric" fluorescence technique using Fura-2 AM. The cells were incubated for 30 min in HEPES buffered Tyrode solution (composition see before) containing also 2 μM dye and then washed. Fluorescence measurements were carried out in modified, UV transparent, temperature-controlled and perfused cell chamber (Cell MicroControls, VA, USA) with a pair of platinum electrodes added for field stimulation. The chamber was attached to the stage of an inverted fluorescence microscope (Diaphot 200, Nikon, Japan). Cells were selected using a red-sensitive video camera system, and were paced at 1 Hz. A 75 W Xenon arc-lamp (Optosource, Cairn, UK) was used for excitation. Excitatory wavelengths (340 nm and 380 nm) were selected via a rapid switching galvanometric monochromator (Optoscan, Cairn, UK). The output of the monochromator was connected to the epifluorescence input of the microscope by a fused silica light guide. Excitatory wavelengths were switched at 100 Hz. Cells were excited through a Fluor 40/0.70 type Nikon objective. Optical signals from the cells were filtered with a band pass filter (425 ± 17.5 nm) and directed into a photomultiplier tube (PMT). An adjustable window was used to restrict the light reaching the PMT in order to minimize background fluorescence from the remainder of the field. The output of the PMT was sampled and demultiplexed at 200 Hz with the Optoscan. Data acquisition and basic processing were performed by a software (Acquisition Engine) supplied with the Optoscan system. Changes in intracellular free calcium levels were approximated by the ratio of the demultiplexed optical signals (340/380) previously corrected against nonspecific background fluorescence.

3.2. Experiments in human cardiac preparations

Hearts were obtained from organ donors whose hearts were explanted to obtain pulmonary and aortic valves for transplant surgery. Before cardiac explantation, organ donor patients did not receive medication, except dobutamine, furosemide, and plasma expanders. The investigations conform to the principles outlined in the Declaration of Helsinki and all experimental protocols were approved by the Albert Szent-Györgyi Medical University Ethical Review Board (No. 51-57/1997). Proper consent was obtained for use of each individual's tissue for experimentation.

3.2.1. Human tissue preparation

Ventricular trabeculae and papillary muscle preparations (< 2 mm in diameter, n =42) obtained from the right ventricles of 24 undiseased human donor hearts (17 male and 7 female, age=45.7±3.8 years). After explantation, each heart was perfused with cardioplegic solution and kept cold (4 – 6 °C) for 2-4 hours prior to dissection. Trabeculae and papillary muscles were then excised and mounted in a tissue chamber (volume ≈ 50 ml) perfused with oxygenated (95% O_2 + 5% CO_2) modified Tyrode's solution containing (in mM): NaCl, 115; KCl, 4; $CaCl_2$, 1.8; MgCl, 1; $NaHCO_3$, 20; and glucose, 11. The pH of this solution was 7.35 to 7.45 at 37 °C.

3.2.2. Experimental techniques: action potential measurements and whole cell patch-clamping

The solutions, equipment and protocols bor both standard microelectrode and patch-clam techniques were in principle similar as used for animal preparations described in detail in the previous paragraph.

3.3. Drugs

SEA-0400 was obtained from Orion Pharma (Espoo, Finland) and was dissolved in 100 % DMSO to make 30 mM stock solution.

Series of new drugs that combine the hydroxy-benzopyran ring of vitamin E with the methylsulfonyl-aminophenyl moiety of class III antiarrhythmic drugs were synthesised and tested. Effects of two families of compounds that were active in ischaemia and reperfusion studies were further analysed in detail on the action potential parameters by applying the

standard microelectrode technique. These compounds are the compound piperazine analogue (5a-5c) and methylamino analogue (19a and 19b) compounds. Figure 1 presents the chemical structure of these compounds. The compounds were dissolved in 100 % DMSO to make 10 mM stock solution.

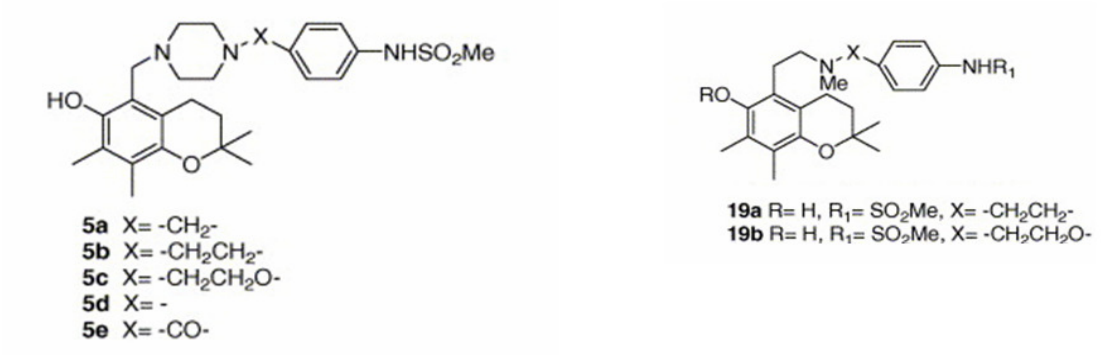

Figure 1. Chemical structures of the piperazine (5a-5e, left panel) and methylamino analogue (19a-19b, right panel) compounds.

I_{Kr} blockers D-sotalol (Bristol-Myers Squibb Co., Wallingford, CT, USA), E-4031 (Institute for Drug Research Ltd., Budapest, Hungary) and dofetilide (synthetised by chemical partner) were prepared daily from aqueous or DMSO stock solutions (30 mM and 1 mM, respectively) to obtain the final drug concentration examined. I_{Ks} blockers chromanol 293B (Aventis Pharma, Frankfurt, Germany), HMR-1556 (Aventis Pharma) and L-735,821 (Merck-Sharpe & Dohme Co, West Point, PA, USA) were similarly diluted from stock solutions (10 mM and 1 mM , respectively) containing 100 % DMSO. This procedure resulted in a 0.01% DMSO concentration when the effects of the drugs were examined. This and the lower DMSO concentrations alone did not affect action potential characteristics in separate studies.

3.4. Statistical analysis

Results were compared using Student's t-tests for paired and unpaired data. Differences were considered significant when $P< 0.05$. Data are expressed as mean ± standard error of the mean (SEM)

4. Results

4.1 The effect of SEA-0400, a newly developed NCX inhibitor devoid of I_{Ca} blocking property, on the NCX and I_{Ca} currents in dog ventricular preparations and Purkinje fibres

4.1.1. Effect of SEA-0400 on the early and delayed afterdepolarizations

Using the conventional microelectrode technique, the effects of SEA-0400 on early (EAD) and delayed (DAD) afterdepolarizations were studied in canine right ventricular papillary muscles and Purkinje fibres, respectively. EADs were evoked in papillary muscle preparations, stimulated at slow cycle lengths (1500 –3000 ms), in the presence of 1 μM dofetilide plus 10 μM $BaCl_2$. The amplitude of the EAD was determined as the difference between the most negative voltage before the appearance of EAD and the peak of the EAD. As Figure 2 shows, 1 μM SEA-0400 decreased the amplitude of EAD. This effect was reversible upon washout of the compound from the tissue bath with solution containing dofetilide and $BaCl_2$. Similar results were obtained in eight additional experiments. The amplitudes of the EADs were decreased by SEA-0400 from 26.6 ± 2.5 to 14.8 ± 1.8 mV in average (n=9; $p<0.05$).

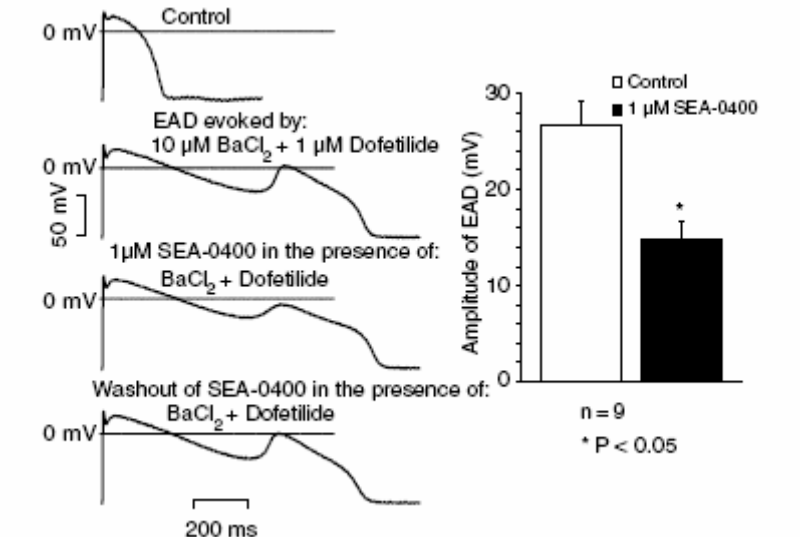

Figure 2. The effect of 1 μM SEA-0400 on EADs in canine cardiac right ventricular muscles is summarized. On the left, the results of a representative experiment are shown; on the right, the average values of the amplitude of EADs are presented before (open bar) and after (filled bar) the administration of SEA-0400.

DADs were evoked in Purkinje fibre preparations superfused with 0.2 μM strophantin for 40 min. In these experiments a train of 40 stimuli was applied at a cycle length of 400 ms,

which was then followed by a 20 s long stimulation-free period in order to generate DADs. These strophantin-induced DADs evoked extra-systoles and automaticity in all six fibres investigated (Figure 3). In three out of the six fibres, DADs were fully abolished by 1 μM SEA-0400. In three fibres, the amplitudes of DADs were decreased by SEA-0400 from 12.5 ± 1.7 to 5.9 ± 1.4 mV ($p<0.05$).

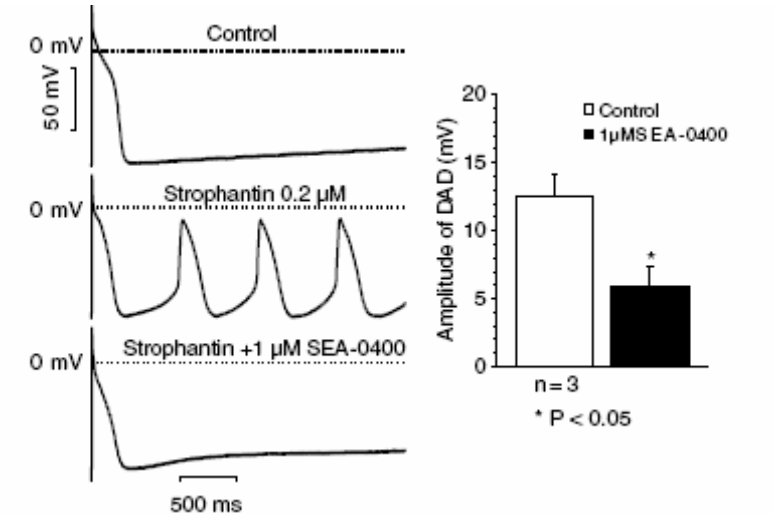

Figure 3. The effect of 1 μM SEA-0400 on DADs in canine cardiac Purkinje fibres is summarized. On the left, results of a representative experiment with triggered activity are shown; on the right, average values of the amplitude of DADs are given before (open bar) and after (filled bar) the application of 1 μM SEA-0400.

4.1.2. Effect of SEA-0400 on L-type Ca^{2+} current and Ca^{2+} transients

Since the selectivity of SEA-0400 on the NCX is an important issue regarding Ca^{2+} handling in this work, we investigated the effects of 1 μM SEA-0400 on I_{Ca} and $[Ca^{2+}]$ in canine myocytes.

I_{Ca} was evoked by 400 ms depolarizing test pulses to 0 mV arising from the holding potential of 40 mV. The interpulse interval was 5 s (0.2 Hz frequency). The amplitude of I_{Ca} was defined as the difference between the peak inward current measured at the beginning of the pulse and the current found at the end of the pulse. As presented in Figure 4, 1 μM SEA-0400 did not change significantly the amplitude of I_{Ca}. The slight decrease of the peak Ca^{2+} current, shown in Figure 4, can be attributed to the run-down of I_{Ca}, since a similar magnitude of decrease was observed in time-matched control measurements.

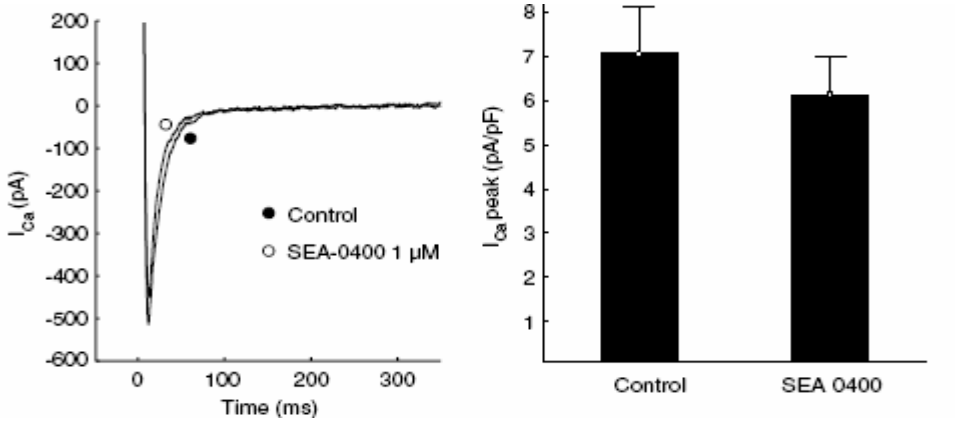

Figure 4. Effect of 1 µM SEA-0400 on the L-type Ca^{2+} current in canine cardiac myocytes. The left panel shows original I_{CaL} current traces from a representative experiment, while right panel summarizes the results obtained from five cells. Bars represent mean ±SEM.

Using the Fura-2-based ratiometric fluorescence technique, no significant changes in the calcium transients could be found. As shown in Figure 5, application of 1 µM SEA-0400 for 5 min changed neither the shape nor the magnitude of the calcium transient. The slight, non-significant decrease in the peak values (to 96.9 ± 6.4% of the control) can well be attributed to slow run-down of the cells.

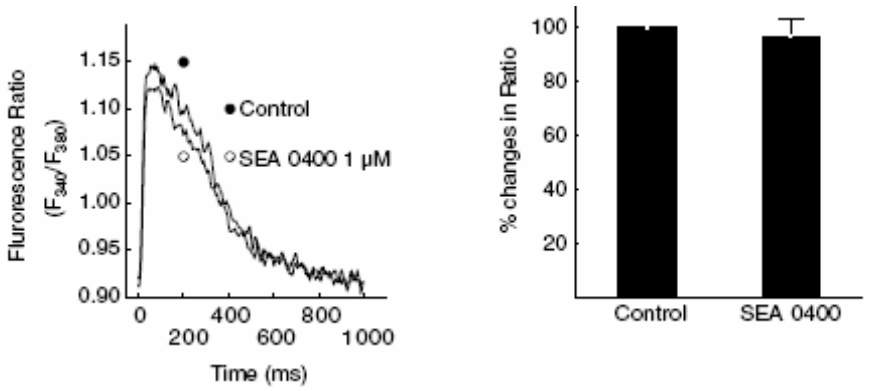

Figure 5. Ratios of fluorescence emission (excitation at 340 and 380 nm, respectively) approximating Ca^{2+} transients before and 5 min following the application of 1 µM SEA-0400 are shown in a representative experiment (left panel) and the statistics for four cells are given in the right panel. Bars represent mean ±SEM.

4.2. Electrophysiological effects of series of molecules that combine the hydroxy-benzopyran ring of vitamin E with the methylsulfonyl-aminophenyl moiety of class III antiarrhythmic drugs

We have tested the effect of a series of some newly synthetised molecules that combine hydroxybenzopyran ring of vitamin E with the methylsulfonyl-aminophenyl moiety of Class III antiarrhythmic drugs on rabbit cardiac preparations. Effects of two families of compounds that were active in ischaemia and reperfusion studies were further analysed in detail on the action potential parameters by applying the standard microelectrode technique. These drugs are the piperazine analogue (5a-5e) and methylamino analogue (19a and 19b) compounds. The effect of compounds 5a–e and 19a,b on the action potential parameters was investigated at 5 μM in rabbit ventricular muscles. The results are summarized in Table 1.

Table 1. Effect of piperazine (5a–5e) and methylamino (19a-19b) analogues, at a concentration of 5 μM, on the action potential parameters in rabbit isolated right ventricular papillary muscle

Compound	**Experiments**	**RP (mV)**	**APA (mV)**	**APD_{50} (ms)**	**APD_{90} (ms)**	**V_{max} (V/s)**
Control	1	–88	122	164	201	201
5a		–88	125	167	201 (0%)	201
Control	2	–89	117	124	165	171
5a		–87	115	133	173 (4.8%)	186
Control	1	–84	110	107	139	186
5b		**–85**	**118**	**110**	**140 (0%)**	**208**
Control	2	–93	114	136	169	156
5b		**–91**	**115**	**136**	**168 (0%)**	**163**
Control	1	–87	121	167	205	223
5c		**–86**	**122**	**188**	**224 (9.3%)**	**230**
Control	2	–77	98	126	175	267
5c		**–84**	**109**	**159**	**199 (13.7%)**	**267**
Control	1	–90	104	157	195	171
5d		**–88**	**100**	**157**	**189 (–3.1%)**	**208**
Control	2	–89	114	112	161	178
5d		**–89**	**112**	**137**	**169 (5%)**	**216**
Control	1	–84	117	127	175	305
5e		**–84**	**117**	**150**	**191 (9.1%)**	**297**
Control	2	–93	113	154	200	163
5e		**–92**	**112**	**167**	**211 (5.5%)**	**171**

Compound	Experiments	RP (mV)	APA (mV)	APD_{50} (ms)	APD_{90} (ms)	V_{max} (V/s)
Control	1	–90	113	197	230	201
19a		**–91**	**120**	**214**	**246 (6.9%)**	**230**
Control	2	–82	105	118	155	193
19a		**–86**	**106**	**127**	**170 (9.7%)**	**260**
Control	1	–86	119	110	150	171
19b		**–86**	**117**	**117**	**160 (6.7%)**	**178**
Control	2	–86	117	199	237	334
19b		**–83**	**103**	**215**	**255 (7.6%)**	**201**

RP = resting membrane potential.
APA = action potential amplitude.
$APD_{50–90}$ = action potential duration at 50% and 90% of repolarization
V_{max} = maximal rate of depolarization.

The compounds did not change the resting potential (RP), the action potential amplitude (APA), and the maximal rate of depolarization (V_{max}). The observed variations of V_{max} in some experiments most likely reflect inconsistency of the impalement of the microelectrode. These data suggest no major change of the fast inward sodium current (I_{Na}) after the application of the analogues. Compounds 5a, 5b and 5d did not alter the repolarization process reflected as no change in the 50% and 90% action potential durations (APD_{50} and APD_{90}) was observed. This indicates the lack of, or minimal effect of the drug on the major repolarizing potassium channels.

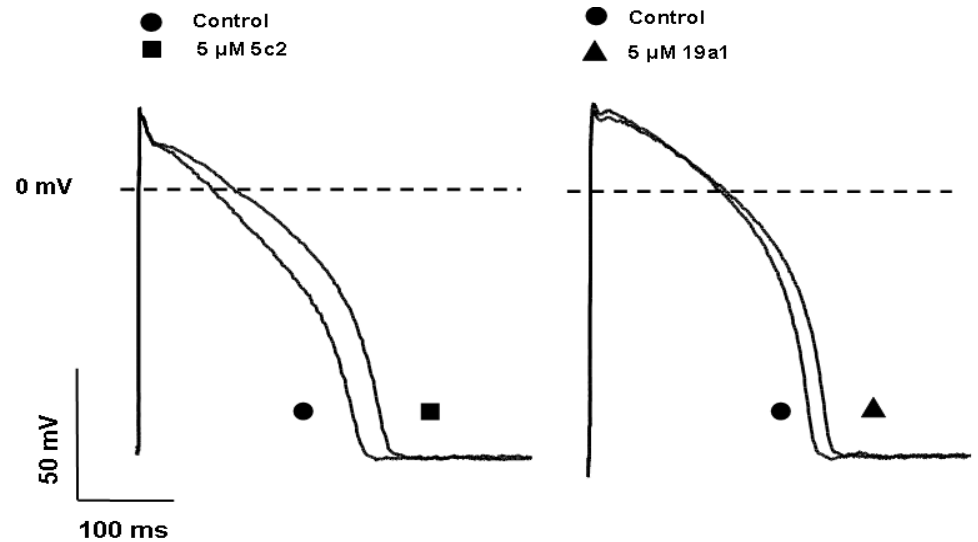

Figure 6. Rabbit ventricular papillary muscle action potential recordings before and after 40 minutes of superfusion with the new molecules 19a1 (5 µM, left) and 5c2 (5 µM, right). Stimulation frequency was 1 Hz.

However, compounds 5c and 5e (Figure 6, left) and 19a and 19b (Figure 6, right) prolonged APD by 5–14%. which suggests moderate inhibition of a repolarizing potassium current, most likely of the rapid delayed rectifier potassium current (I_{Kr}). The latter effect represents a moderate Class III antiarrhythmic action and may need further investigations.

4.3. In vitro electrophysiological effects of SZV-123, a new amiodarone-like (combined Class I/B+III) antiarrhythmic drug, on the action potential parameters and main repolarizing transmembrane currents in dog and rabbit cardiac preparations.

We have tested the effect of newly synthesised amiodarone-like antiarrhythmic drugs on dog and rabbit cardiac preparations. The SZV-123 compound significantly lengthened the action potential duration (APD) on ventricle, atrium and Purkinje fibre of the dog and rabbit and cardiac myocytes (Figure 7 and Tables 2-5). This phenomenon indicates a Class III antiarrhythmic repolarization blocking effect.

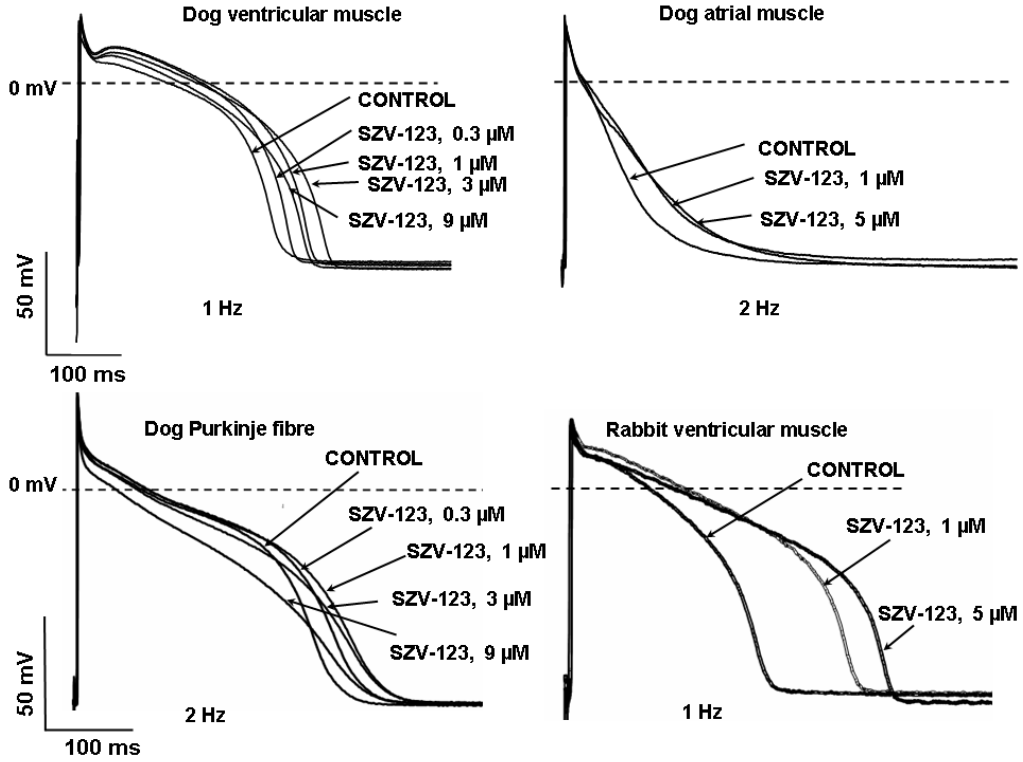

Figure 7. Concentration-dependent effects of the new amiodarone-like (combined Class IB+III) compound SZV-123, on the action potential duration recorded in preparations from dog ventricle (upper left) and atria (upper right), from dog Purkinje fibre (bottom left) and from rabbit ventricle (bottom right). Respective stimulation frequencies are given on the figure.

Besides this effect, SZV-123 in the 0.3–0.9 μM concentration range blocked the maximal rate of depolarization (V_{max}) in a concentration dependent manner, which also suggests a Class I/B type sodium channel blocking effect (Tables 2-5). It is important to note that SZV-123 at concentration higher than 3 μM did not lengthen the repolarization on the Purkinje fibres.

Table 2. The effect of SZV-123 on the action potential parameters in rabbit ventricular muscle preparation

n=5	**RP mv**	**APA mV**	**APD_{25} ms**	**APD_{50} ms**	**APD_{90} ms**	**V_{max} V/s**
Control	-89.0 ± 0.8	115.6 ± 3.9	68.4 ± 8.6	115.8 ± 12.6	156.6 ± 10.2	252.6 ± 21.9
SZV-123						
1 μM	-88.4 ± 0.5	116.6 ± 3.2	70.4 ± 9.0	145.8 ± 20.7*	199.8 ± 18.9*	271.4 ± 30.5
5 μM	-88.0 ± 0.8	112.0 ± 2.3	67.2 ± 11.2	164.6 ± 34.8*	226.8 ± 32.6*	260.2 ± 23.1

Frequency of stimulation = 1 Hz
RP = resting potential
APA = amplitude of the action potential
$APD_{25–50–90}$ = action potential duration at 25, 50 and 90% of repolarization
V_{max} = maximal rate of depolarization
ERP = effective refracter period
*= $p < 0.05$

Table 3. The effect of SZV-123 on the action potential parameters in dog ventricular muscle preparation

n=5	**RP mV**	**APA mV**	**APD_{50} ms**	**APD_{90} ms**	**V_{max} V/s**	**ERP ms**	**ERP/APD**
Control	-85.0 ± 1.2	116.2 ± 2.5	182.2 ± 7.1	222.6 ± 13.2	296.0 ± 30.7	215.8 ± 10.2	0.973 ± 0.02
SZV-123							
0.3 μM	-85.8 ± 1.5	116.6 ± 1.8	210.6 ± 155*	252.2 ± 14.2*	288.4 ± 24.3	243.6 ± 10.9*	0.969 ± 0.05
1 μM	-86.2 ± 1.2	116.2 ± 2.8	232.6 ± 17.6*	277.4 ± 16.6*	292.8 ± 37.4	267.2 ± 14.7*	0.965 ± 0.01
3 μM	-86.4 ± 1.0	120.4 ± 1.3	242.8 ± 19.5*	295.8 ± 18.4	300.0 ± 33.7	289.4 ± 15.6	0.980 ± 0.01
9 μM	-87.2 ± 1.0	117.8 ± 1.6	222.4 ± 19.2*	275.8 ± 19.6*	285.6 ± 30.6	267.0 ± 17.9*	0.970 ± 0.01

Frequency of stimulation = 1 Hz

Table 4. The effect of SZV-123 on the action potential parameters in dog Purkinje fibre

n = 7	RP mV	APA mV	APD_{50} ms	APD_{90} ms	V_{max} V/s	ERP ms	ERP/APD
Control	-85.3 ± 1.1	127.6 ± 2.7	168.3 ± 12.3	241.4 ± 4.3	773.0 ± 53.2	221 ± 4.1	0.918 ± 0.01
SZV-123							
0.3 μM	-85.0 ± 0.9	125.9 ± 3.0	176.9 ± 13.9	266.1 ± 3.5*	680.4 ± 69.3	248.7 ± 3.8	0.935 ± 0.01
1 μM	-85.7 ± 0.5	126.6 ± 2.0	186.6 ± 8.0*	285.9 ± 5.2*	672.1 ± 54.5	268 ± 7 ± 5.2	0.940 ± 001
3 μM	-85.3 ± 0.7	124.7 ± 2.3	160.9 ± 8.0	279.9 ± 6.2*	643.3 ± 62.6	259.7 ± 6.1	0.928 ± 0.01
9 μM	-85.1 ± 0.7	123.1 ± 3.6	116.6 ± 8.1*	252.9 ± 14.4	664.7 ± 46.6	234.1 ± 15.4	0.924 ± 0.01

Frequency of stimulation = 2 Hz

Table 5. The effect of SZV-123 on the action potential parameters in dog atrial muscle preparation

n = 6	RP mV	APA mV	APD_{25} ms	APD_{50} ms	APD_{90} ms	V_{max} V/s
Control	-87.5 ± 1.4	112.0 ± 2.7	27.8 ± 2.7	52.8 ± 2.9	86.6 ± 3.1	347.8 ± 55.8
SZV-123						
1 μM	-87.3±1.3	114.2 ± 1.5	30.3 ± 2.8	63.0 ± 4.6*	105.2 ± 3.8*	322.3 ± 9.6
5 μM	-88.0 ± 0.4	111.0 ± 3.0	28.8 ± 4.2	60.7 ± 6.3	107.4 ± 6.9*	275.3 ± 41.7

Frequency of stimulation = 2 Hz

We have tested the effects of the SZV-123 on the main repolarizing transmembrane currents in ventricular myocytes isolated from dog hearts by applying the whole-cell patch-clamp technique at 37 °C.

I_{Kr} was activated by 1 s long depolarizing test pulses to membrane potentials ranging from -20 mV to +50 mV. The interpulse interval was 20 s (0.05 Hz frequency) to allow the complete deactivation of I_{Kr}. The amplitude of the tail current measured upon returning to the holding potential of -40 mV was plotted as a function of the activation voltage and was used to define the magnitude of I_{Kr}. These experiments were performed in the presence 100 nM L-735,821 in order to eliminate I_{Ks}. The drug almost completely blocked the fast delayed rectifier potassium current (I_{Kr}), even at the low concentration of 1 μM, which suggest a strong HERG channel inhibition (Figure 8). This effect looked reversible, since the current re-

increased (Figure 8, left bottom panel) after the drug was washed-out from the bath.

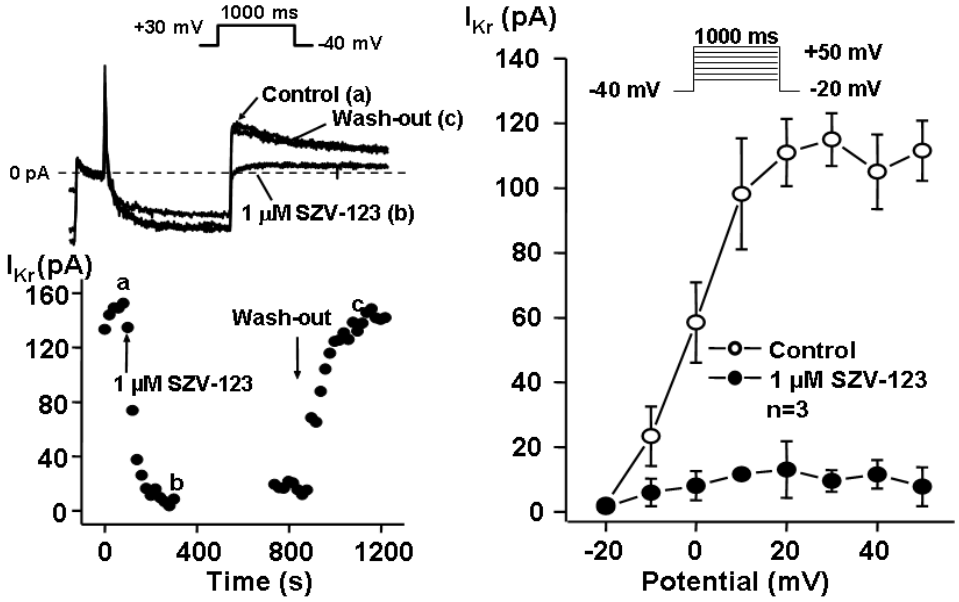

Figure 8. The effect of 1 μM SZV-123 on the rapid component of the delayed rectifier outward potassium current (I_{Kr}) in single dog ventricular myocyte. Panel A shows original recordings from a representative experiment. Panel B indicates the current-voltage relation before (open circle) and after (closed circle) SZV-123 (1 μM for 3-5 minutes) superfusion in an average of 3 cells. Insets show applied voltage protocols. Bars indicate ±SEM.

Transient outward current (I_{to}) was evoked by applying 400 ms long test depolarizations to voltages ranging from -10 to +60 mV, preceded by a 5 ms short prepulse to -40 mV (for inactivation of fast I_{Na}). These depolarizations were arising from the holding potential of -90 mV, and were separated by 3 s long (0.33 Hz frequency) interpulse intervals. Figure 9 shows that SZV-123 did not affect the amplitudes of the I_{to} even at the high concentration of 10 μM.

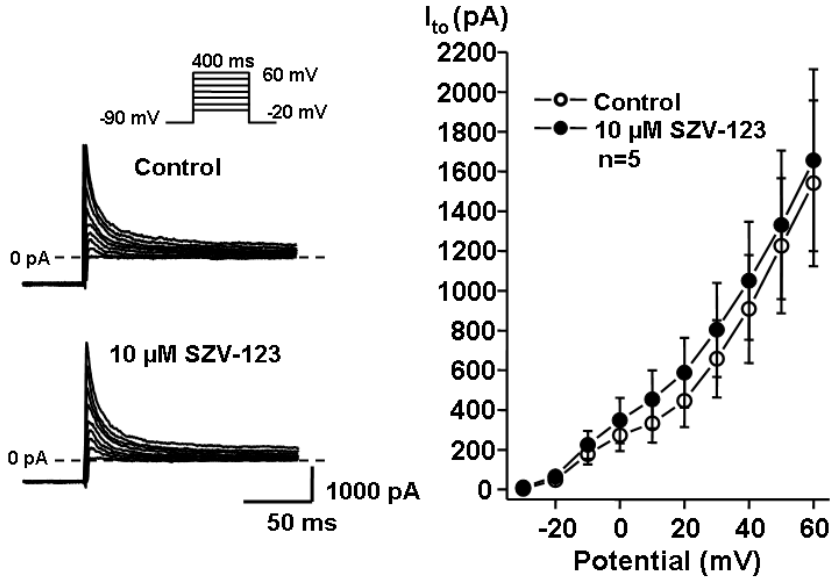

Figure 9. Lack of effect of 10 μM SZV-123 on the transient outward current (I_{to}) in single dog ventricular myocytes. Panel A shows original recordings from a representative experiment. Panel B indicates the current-voltage relation before (open circle) and after (closed circle) SZV-123 (10 μM for

3-5 minutes) superfusion in an average of 5 cells. Inset shows applied voltage protocols. Bars indicate ±SEM.

Steady-state current-voltage relationship of the membrane was determined by applying 400 ms long voltage pulses to test potentials ranging from -130 mV to 0 mV, arising from the holding potential of -90 mV, and separated by 3 s long (0.33 Hz frequency) interpulse intervals. Membrane currents measured at the end of these pulses were plotted against their respective test potentials. The negative branch of the I-V curve is associated with inward rectifier potassium current (I_{K1}). Figure 10 shows that SZV-123 did not affect the amplitude of I_{K1} currents even at the high concentration of 10 μM.

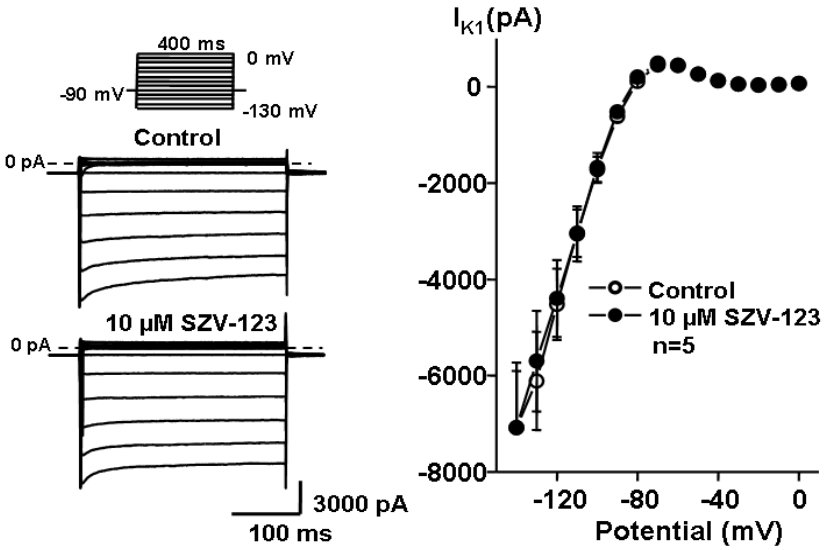

Figure 10. Lack of effect of 10 μM SZV-123 on the inward rectifier potassium current (I_{K1}) in single dog ventricular myocytes. Panel A shows original recordings from a representative experiment. Panel B indicates the current-voltage relation before (open circle) and after (closed circle) SZV-123 (10 μM for 3-5 minutes) superfusion in an average of 5 cells. Inset shows applied voltage protocols Bars indicate ±SEM.

4.4. The role of slow delayed rectifier potassium current (I_{Ks}) in human ventricular muscle

4.4.1. Effects of I_{Ks} and I_{Kr} blockade on normal human ventricular muscle action potential duration in the absence of sympathetic stimulation

Concentrations of chromanol 293B (10 μM), L-735,821 (100 nM) and HMR-1556 (100 nM and 1 μM) that were reported to selectively block I_{Ks} in cardiac ventricular muscle preparations in other species [39, 54] produced little (<9 ms, 2.8 %) change in human ventricular papillary muscle APD after 40 minutes of exposure during continuous pacing at a cycle length of 1000 ms (Figure 11, top and middle panels). In contrast, concentrations of d-sotalol (30 μM) and E-4031 (1 μM) expected to selectively block I_{Kr} [39, 47, 54], markedly

and significantly increased human ventricular muscle APD under identical conditions (Figure 11, bottom panels).

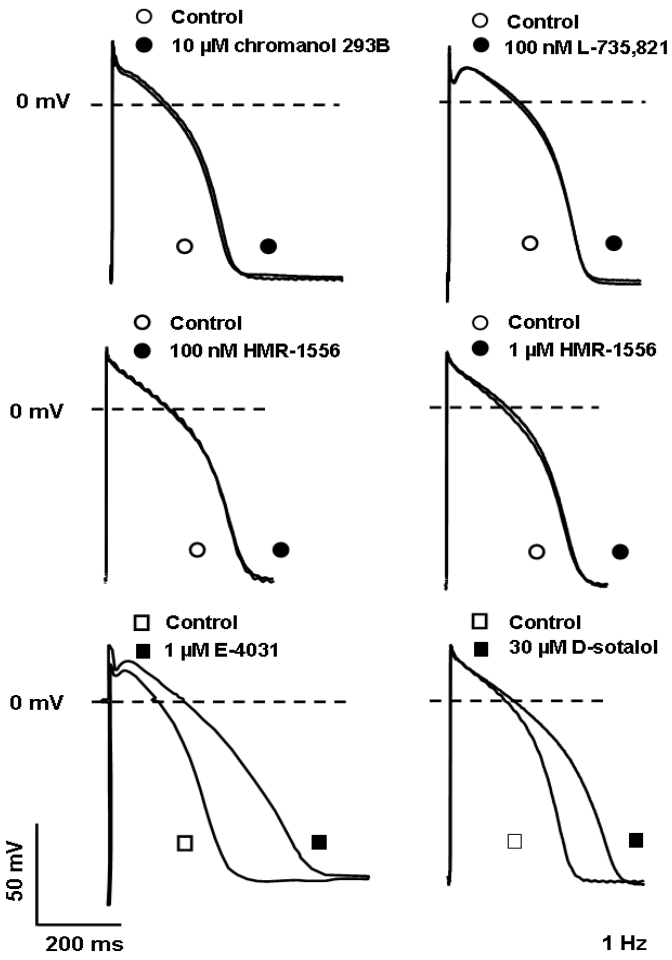

Figure 11. Human ventricular papillary muscle action potential recordings in the absence of any sympathetic agonist before and after 40 minutes of superfusion with the I_{Ks} blocker 10 µM chromanol 293B (top left), 100 nM L-735,821 (top right) 100 nM and 1 µM HMR-1556 (middle), and the I_{Kr} blockers 1 µM E-4031 (bottom left) and 30 µM d-sotalol (bottom right). Stimulation frequency was 1 Hz.

This difference in the effects of chromanol 293 B (10 µM), L-735,821 (100 nM) and HMR 1556 (100 nM and 1 µM) compared to d-sotalol (30 µM) and E-4031 (1 µM) on APD was observed in human ventricular muscle over a wide range of pacing cycle lengths (300 to 5000 ms) (Figure 12). Over this range of pacing cycle lengths, chromanol 293 B (Figure 12), L-735,821 or HMR (100 nM and 1 µM) produced a change of ≤12 ms (3.2 %) in APD, whereas d-sotalol and E-4031 each markedly lengthened human ventricular APD in a reverse frequency-dependent manner.

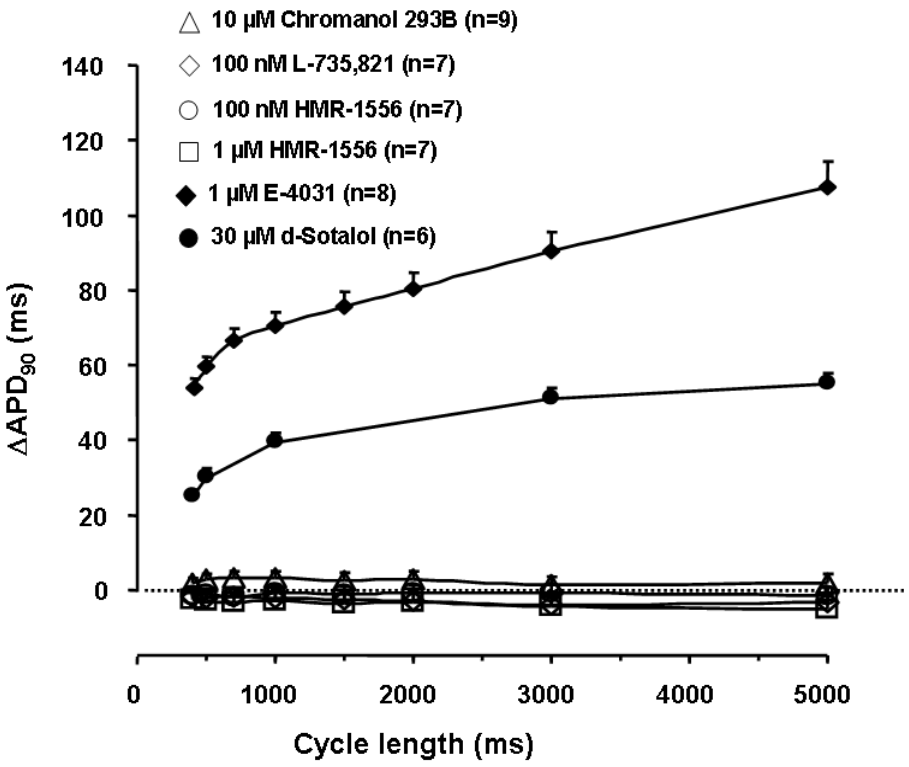

Figure 12. Frequency dependent effect of I_{Kr} (by 1 µM E-4031, or 30 µM sotalol) and I_{Ks} block (by 10 µM chromanol 293B, 100 nM and 1 µM HMR-1556 or 100 nM L-735,821) on action potential duration (APD) in canine ventricular papillary muscles in the absence of any sympathetic agonist. Abscissa = Pacing cycle length; ordinate = percentile changes in APD_{90}. Bars represent ± SEM

4.4.2. The effect of I_{Ks} block during increased sympathetic activation following attenuation of repolarization reserve

The effect of 1 µM HMR-1556 was tested also in preparations, where the repolarization reserve was initially attenuated by selective I_{Kr} block with 50 nM dofetilide, and sympathetic stimulation activated by 1 µM adrenaline. In these experiments, HMR-1556 induced I_{Ks} block significantly lengthened APD (14.7±3.2 %, $p<0.05$, n=3; Figure 13). This effect is in sharp contrast to the negligible effect of HMR-1556 on normal APD (Figures 11 and 12), and indicates that the effect of I_{Ks} on repolarization is substantially increased when sympathetic activation is increased and when a reduction in repolarization reserve results in an abnormally long APD.

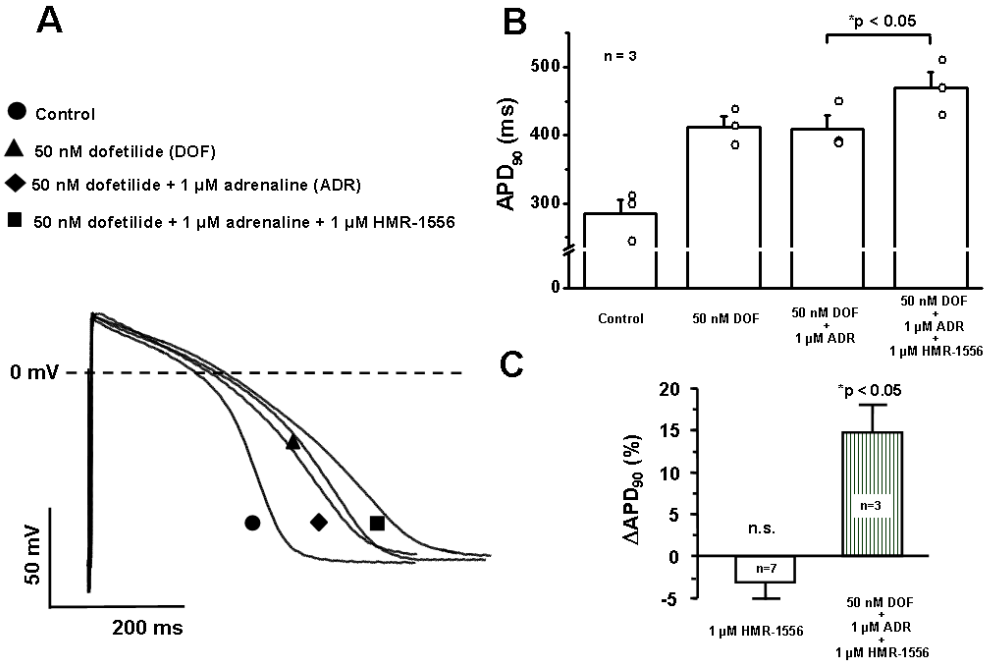

Figure 13. Effect of 1 µM HMR-1556 on human ventricular action potentials recorded in the presence of 50 nM dofetilide (DOF) and 1 µM adrenaline (ADR) to reduce repolarization reserve. Panel A. Representative action potentials recorded at baseline (●), following exposures to 50 nM dofetilide (▲), 50 nM dofetilide +1 µM adrenaline (♦), and after addition of 1 µM HMR-1556 in the continued presence of dofetilide and adrenaline (■). Panel B. Similar effects on APD averaged in three experiments. Bars show mean ±SEM, while circles represent each individual measurement. Panel C. Comparison of the effect of 1 µM HMR-1556 on normal (open bars, similar data from Figures 11 and 12) and on action potentials recorded in the presence of 50 nM dofetilide and 1 µM adrenaline (striated bars, similar data from panel B). Columns and error bars indicate means±SEM. Significant changes ($p<0.05$, n=3) between the conditions represented by the bars.

4.5. Contribution and relative role of the rapid delayed and inward rectifier potassium channels (I_{Kr} and I_{K1}) in human, dog and rabbit ventricular repolarization

In isolated left ventricular myocytes obtained from human, dog and rabbit hearts the density of the I_{K1} and I_{Kr} currents were compared. Figure 14A shows the corresponding I-V curves of the I_{K1} currents from isolated human, dog and rabbit myocytes. I_{K1} current was measured by applying similar voltage protocols as applied for investigation of the effects of SZV-123 compound described earlier in detail. These curves indicate that the I_{K1} current amplitude was significantly larger in dog and rabbit than in human ventricular cells in the voltage range between –70 and –50 mV (Figure 14A, top). There was, however, no significant difference in the I_{K1} amplitude in the voltage range between –30 and 0 mV. The maximal current amplitude at –60 mV was significant larger in dog and rabbit myocytes (almost three-five times) than that measured in human cells (Figure 14A, bottom).

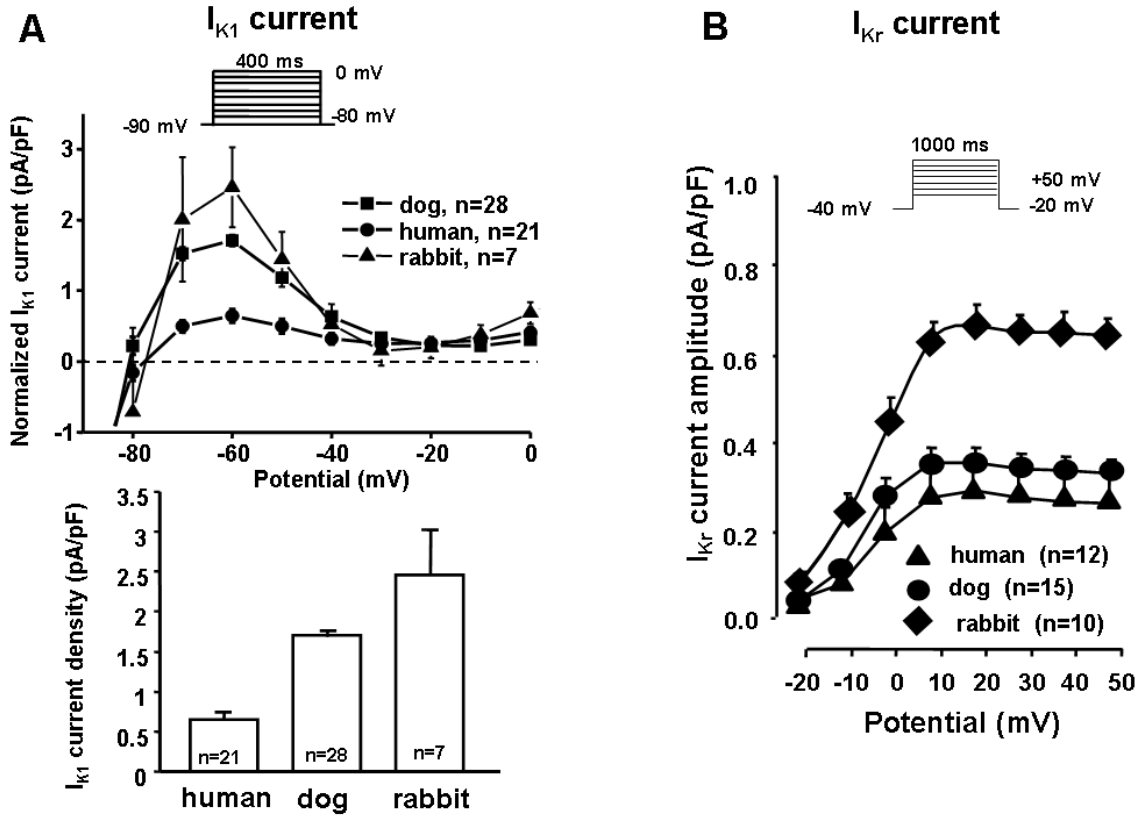

Figure 14. Panel A. Current-voltage relationships of I_{K1} current in human, dog and rabbit ventricular myocytes (top). I_{K1} current densities measured at -60 mV membrane potential (bottom). Panel B. Current-voltage relationships of I_{Kr} tail current in human, dog and rabbit ventricular myocytes. Insets show applied voltage protocols. Values and bars represent mean±SEM.

Figure 14B illustrates the I-V curves of the I_{Kr}-tail currents in human, dog and rabbit ventricular myocytes (Figure 14B). I_{Kr} current was measured by applying similar voltage protocols as applied for investigation of the effects of SZV-123 compound described earlier in detail. The diagram shows that the amplitude of I_{Kr} measured at –40 mV as tail current after 1000 ms test pulses between –20 and –50 mV did not significantly differ from each other in human and dog ventricular cells, while in rabbit, the amplitude of I_{Kr} tails was almost double of that observed in dog and human.

Although the activation and deactivation kinetics of I_{Kr} in human and dog differ from each other (not shown) (activation τ in human at +30 m 36.6±3.2 ms, n=6 *vs.* 53.8±5.8 ms, n=15 in dog, and deactivation τ (at –40 mV in human τ_1=600±53.9 ms and τ_2=6792±875 ms, n=5 vs. τ_1=360.3±26.3 ms and τ_2=3310±280 ms, n=15 in dog) but these modest differences were not expected to cause substantially different I_{Kr} densities during the time course of the action potential at normal heart rate.

The impact and contribution of I_{K1} and I_{Kr} to the repolarization was studied by investigating the effect of 10 µM $BaCl_2$ and 1 µM E-4031 on the action potential duration by applying the standard microelectrode technique in ventricular preparations from dog, rabbit and human hearts. 10 µM $BaCl_2$ and 1 µM E-4031 are considered to selectively block I_{K1} and I_{Kr} currents, respectively [39, 40]. In human ventricular muscle inhibition of I_{K1} by 10 µM $BaCl_2$ elicited only marginal prolongation of the action potential duration, while it caused significant prolongation of the action potential duration in the dog and rabbit papillary muscle (18.6±4.3% in rabbit, 17.9 ± 2.1 % in dog and 4.8 ± 1.5 % in human, respectively, n=7-11, Figures 15A and 15B, left).

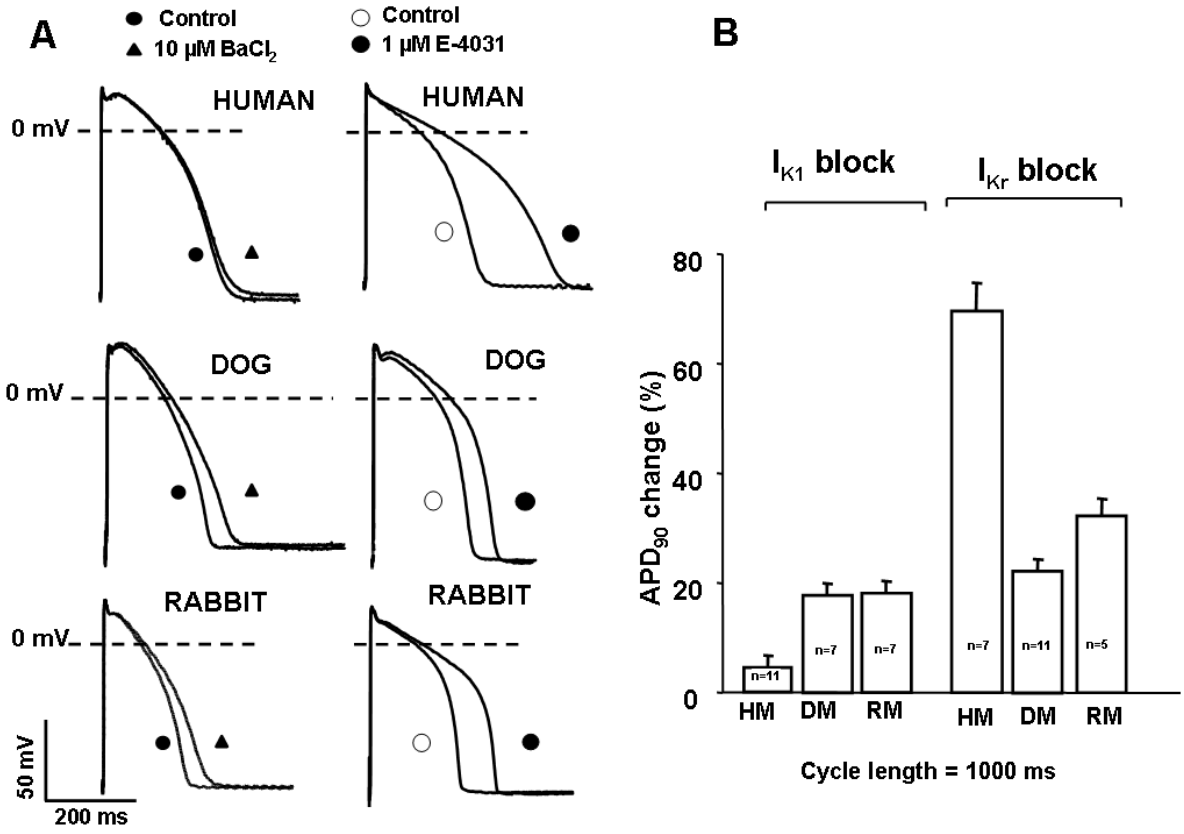

Figure 15. Panel A. Effect of selective I_{K1} (10 µM) and I_{Kr} (1 µM E-4031) block on the APD recorded in intact right human, dog and rabbit papillary muscle action potentials. Panel B. Effect of selective I_{K1} (10 µM) and I_{Kr} (1 µM E-4031) block on the APD_{90} changes in human (HM), dog (DM) and rabbit (RM) papillary muscles. Stimulation frequency was 1 Hz.

On the contrary, inhibition of I_{Kr} by E-4031 in the contrary, caused significantly larger action potential duration prolongation in the human (up to 70 %) than in the dog and rabbit papillary muscle (70.2 ± 4.1 % in human, 22.8 ± 2.5 % in dog and 35.4 ± 3.8 % in rabbit, respectively, n=6-8, Figures 15A and 15B, right).

5. Discussion

Multicellular electrophysiological recording techniques have turned out to be the cutting edge of scientific research methods again, since single cell systems have several shortcomings (deleterious effects of cell isolation on the transmembrane ion channels are poorly understood). Consequently, physiological conditions are not adequate enough for measuring transmembrane action potentials, which are always a result of a fine balance of these transmembrane ion currents. Thus, methods using intracellular microelectrodes to measure action potentials are having their renaissance recently. Using this technique the sum of the inward and outward ion currents (action potential) can be measured in a contracting heart muscle preparation without the interfering with their intracellular environment. Changes in parameters of the action potential while studying the effects of different drugs affect changes occurring in different ion currents. This method is stable and reliable, which appears to be its main advantage.

The specific changes in the individual ion currents can be best measured using the patch-clamp technique. However, when measuring the sum of these currents, *i.e.* the action potential, this method has the above mentioned shortcomings. Therefore, in my research project action potentials were always measured by the conventional intracellular microelectrode and transmembrane ion currents by the whole cell configuration of the patch-clamp technique.

5.1. The electrophysiological effects of SEA-0400, a newly developed NCX inhibitor devoid of I_{Ca} blocking property, on the NCX and I_{Ca} currents in dog cardiac preparations

In the present study, the effect of SEA-0400, a new and potent selective inhibitor of the NCX, was investigated on early and delayed afterdepolarizations in canine ventricular papillary muscles and Purkinje fibres. It was reported that SEA-0400 inhibited the NCX current in dog ventricular myocytes at relatively low concentrations with an estimated average IC_{50} of 0.108 μM for the outward, and 0.111 μM for the inward NCX current [58].

The main finding of this study was that SEA-0400 effectively decreased the amplitude of EADs and DADs evoked in dog ventricular papillary muscle and cardiac Purkinje fibres, respectively. In the same preparations SEA-0400 even at the high concentration of 1 μM, did not influence either I_{Ca} current measured by the patch-clamp or fast I_{Na} current determined as

V_{max} by the conventional microelectrode techniques. Consequently, decrease of inward currents and thereby diminution of Ca^{2+} load via the I_{Ca} and I_{Na} can not explain the effect of SEA-0400 on EAD and DAD amplitude. Our unpublished data (not shown) indicate that SEA-0400 does not change the inward rectifier potassium current (I_{K1}) and neither, or slightly decreases the rapid delayed rectifier (I_{Kr}) and transient outward (I_{to}) potassium currents, respectively. Therefore, participation of the major potassium currents is also unlikely in the mechanism whereby SEA-0400 decreases EADs and DADs, although possible involvement of other transmembrane ionic currents, such as I_{Cl} and Na/K pump current, can not be completely ruled out.

The only study which has recently described specific inhibition of the NCX current by the new compound, SEA-0400, does not include the examination of the effect of this compound on arrhythmogenesis, i.e. formation of EAD and DAD, on action potentials and contractility. KB-R7943 was reported to decrease NCX current and abolished experimental arrhythmias [59]. However KB-R7943 can not be considered as a specific inhibitor of NCX current since at micromolar range depresses the L-type calcium current as well. Therefore, our present investigation is the first which directly addresses the question whether specific NCX inhibition results in suppression of triggered arrhythmias in *in vitro* cardiac preparations.

The possible therapeutic implication of our study appears to be rather complex. It is tempting to speculate that suppression of EADs and DADs may be antiarrhythmic both in the ventricles and the atria [60] during Ca^{2+} overload such as in heart failure and at the beginning of atrial flutter and fibrillation, especially when potassium currents may have been downregulated [61, 62] and the NCX current upregulated [63]. In other experiments in chronic fibrillation in the goat where I_{K1} is upregulated, inhibition of Na^+/H^+ pump and NCX current was not beneficial [64]. Also, it was considered that during reperfusion after myocardial ischaemia, Ca^{2+} influx occurs via the NCX in the reverse mode contributing to Ca^{2+} overload and triggering the release of Ca^{2+} from the sarcoplasmic reticulum and thereby causing cardiac arrhythmias [65]. Thus, blocking the reverse portion of the NCX current can also be beneficial. In addition, the positive inotropic effect of the inhibition of the forward mode of the NCX current can improve myocardial perfusion and, as such, it can be also beneficial. By specific NCX inhibition, unlike PDE inhibition, catecholamines and various calcium current activations, positive inotropic effect can be achieved without harmful electrophysiological consequences, *i.e.* causing depolarization leading to EADs or DADs.

On the other hand, elevated intracellular Ca^{2+} concentration and enhanced cardiac force development may increase myocardial oxygen consumption, and if the Ca^{2+} extrusion

systems other than NCX [66, 67] can not properly compensate, the resulting excessive Ca^{2+} overload may affect the mitochondria and eventually may cause irreversible myocardial damage.

5.2. Electrophysiological effects of series of molecules that combine the hydroxy-benzopyran ring of vitamin E with the methylsulfonyl-aminophenyl moiety of class III antiarrhythmic drugs

The aim of this work was to design, synthetize and test the effect of series new drugs that has powerful antiarrhythmic potential without proarrhythmic side effects. One possible starting point was that several Class III antiarrhythmic drugs as ibutilide, azimilide, sotalol, dofetilide posses a strong antiarrhythmic potency in suppressing ventricular fibrillation, but they administration in the clinical setting is hampered by their relatively large proarrhythmic potency also [27-29]. In this work we have synthetised and tested the effect of a series of new molecules that combine the hydroxybenzopyran ring of vitamin E with the methylsulfonyl-aminophenyl moiety of Class III antiarrhythmic drugs on rabbit cardiac preparations. Effects of two families of compounds that were active in ischaemia and reperfusion studies were further analysed in detail on the action potential parameters by applying the standard microelectrode technique. These drugs were the piperazine analogue (5a-5e) and methylamino analogue (19a and 19b) compounds. Among piperazine derivatives, compounds 5c and 5e suppressed reperfusion tachycardia, while compound 5e reduced premature beats and MDA content, combining antiarrhythmic and antioxidant properties. The presence of the phenyl piperazine moiety (analogue 5d) abolished antiarrhythmic activity. The methylamino derivative 19a exhibited antioxidant activity and reduced premature beats, and induced a fast recovery of the heart during reperfusion. Compounds 5c, 19a and 19b facilitated the recovery of QRS and QT intervals during reperfusion, to the normal values. Moreover, the cardioprotective compounds 5c-5e and 19a-19b did not induce excessive lengthening of the action potential, exhibiting moderate Class III antiarrhythmic actions. Further studies on animal models as well as on the possible influence on specific potassium channels such as I_{Kr}, I_{Ks}, I_{to}, and I_{K1} should clarify the mechanism and provide additional evidence of efficacy of these compounds.

5.3. *In vitro* electrophysiological effects of SZV-123, a new amiodarone-like antiarrhythmic drug, on the action potential parameters and main repolarizing transmembrane currents in dog and rabbit cardiac preparations.

The purpose of the next set of experiments was to examine the cellular electrophysiological effects of SZV-123, a new amiodarone-like antiarrhythmic compound that combines Class I/B+III actions, in isolated dog and rabbit cardiac muscle. The most important result of this study was that SZV-123 lengthened repolarization in a frequency independent manner, and decreased V_{max} only at heart rates faster than the normal range. This is an advantageous characteristic, because an excessive (extreme) repolarization prolongation may lead tos severe arrhythmias (proarrhythmic and torsadogenic side effect) [34]. This property of SZV-123 can be related to the sodium channel blocking effect of the compound that was verified with the observed action potential triangulation on the Purkinje fibres (Figure 7, left bottom panel). The frequency dependent Class I type sodium channel blocking of SZV-123 can be associated with a mexiletine-like fast restoration kinetics (Class I/B effect) [68], therefore it can be expected to decrease the conduction velocity only at frequencies higher than physiological.

It can be concluded that SZV-123 indeed possesses an amiodarone-like multi-channel blocking (Class I/B + III) antiarrhythmic effect, therefore this molecule is promising for treatment of ventricular arrhythmias. Further *in vitro* and *in vivo* studies need to test its efficacy in cardiac arrhythmias. An important issue is to test wether this compound is indeed devoid of proarrhytmic side effects.

5.4. The role of slow delayed rectifier potassium current (I_{Ks}) in human ventricular muscle

Our results indicate that in isolated, multicellular ventricular muscle obtained from normal, undiseased human hearts neither chromanol 293B nor L-735,821 and HMR-1556 markedly increased action potential over a range of pacing cycle lengths corresponding to heart rates of 12 to 200 BPM in the absence of a sympathetic agonist. In addition, our studies indicate that the concentrations of chromanol 293B and L-735,821 (10 μM and 100 nM, respectively) that failed to increase APD significantly blocked I_{Ks} in ventricular myocytes isolated from the same normal, undiseased human hearts. In contrast to these findings, we demonstrated that E-4031 (1 μM) blocked I_{Kr} and dramatically increased normal human

ventricular muscle APD, as did sotalol (30 μM), another recognized I_{Kr} blocker that also dramatically increased human ventricular muscle APD under the same conditions that chromanol 293B and L-735,821 failed to.

The only study before this one to investigate the effect of chromanol on human ventricular action potential characteristics was performed in right ventricular myocytes isolated from hearts explanted from patients with end-stage heart failure [69]. In that study, 1 to 10 μM chromanol 293B was reported to significantly increase APD [69]. However, when Schreieck *et al.* examined the effects of 10 μM chromanol in guinea pig ventricular muscle preparations, they found no effect on APD in the absence of ß-adrenoceptor stimulation [70]. When we previously examined the effects of chromanol 293B in rabbit [54] and dog [39] ventricular papillary muscle preparations, 10 μM chromanol 293B did not significantly increase APD. In our previous studies we also demonstrated that the other I_{Ks} blocker, L-735,821 (100 nM), also did not significantly affect APD in the absence of a sympathetic agonist [39, 54].

The explanation for these differences in results is unclear; although some investigators [71] have attributed them to differences between single cell and multicellular preparations. Sun *et al.*, for instance, reported that higher chromanol concentrations (30-100 μM) lengthened APD in perfused, multicellular dog ventricular muscle "wedge" preparations [72]. Based on this finding, they speculated that chromanol is less able to diffuse into multicellular preparations than into single cells, and that its effects are therefore less pronounced in multicellular preparations. Thus, this group argues [71, 72] that higher concentrations of chromanol are necessary in multicellular ventricular preparations to achieve I_{Ks} block and increase APD than are necessary to completely block I_{Ks} in isolated myocytes. Other investigators also suggested that because of its physical and/or chemical properties, L-735,821 poorly penetrates multicellular preparations but easily enters single myocytes (Dr. J.J. Salata, unpublished personal communication, 1998).

These explanations appear unlikely. In the present study in human, and in our previous studies with rabbit [54] and dog [39], ventricular muscle, a concentration of L-735,821 and HMR-1556 that fully blocked I_{Ks} in single myocytes also failed to increase APD in multicellular preparations; although L-735,821 and HMR-1556 are reportedly more specific and potent than chromanol 293B [73, 74]. In addition, the L-735,821 and HMR-1556 concentrations used in our experiments, 100 nM and 1 μM, respectively, are reported to be more than 10 times their EC_{50} for I_{Ks} block. Furthermore, concentrations of E-4031 and d-sotalol that block I_{Kr} in isolated myocytes markedly increased APD in multicellular preparations; it appears unlikely that either

E-4031 or sotalol have distinctly different abilities to penetrate multicellular preparations and single myocytes than does L-735,821.

On the other hand, Stengl *et al*. [55] and Volders *et al.* [56] recently obtained results similar to our finding that I_{Ks} block does not affect normal ventricular muscle action potential duration in species other than guinea pig in the absence of sympathetic stimulation. They reported that in both dog ventricular myocytes and papillary muscle preparations, HMR-1556 (a highly selective I_{Ks} blocker) failed to lengthen APD without prior sympathetic simulation [55, 56] even at high concentrations. These authors concluded that I_{Ks} block induced repolarization lengthening requires an elevated degree of sympathetic tone as may occur in the setting of heart failure. Others [75] have suggested that the sensitivity of action potential duration shortening by I_{Ks} block is enhanced when phosphorylation is increased as expected during increased sympathetic nerve activity. Clearly, cAMP increases I_{Ks}, and it may alter activation and deactivation kinetics for I_{Ks}. Thus, I_{Ks} is expected to have different effects on APD when sympathetic tone is increased. This relation between phosphorylation and the effects of I_{Ks} block on ventricular muscle APD needs further investigation to better elucidate the importance of sympathetic neural influences on electrogenesis in normal and diseased human myocardium. Nonetheless, our present findings clearly indicate that neither chromanol 293B nor L-735,821 and HMR-1556 markedly affect normal human ventricular muscle APD over a normal range of heart rates in the absence of sympathetic stimulation. Thus, I_{Ks} in the absence of sympathetic neural agonists plays little role in the repolarization of normal ventricular muscle action potentials. Rather, in normal human ventricular myocardium, I_{Kr} is the outward current most responsible for termination of the action potential plateau and initiation of final action potential repolarization. However, as we have previously speculated, I_{Ks} may likely play a vital role in normal myocardium when APD is prolonged as following a pause in rhythm, decreased levels of thyroid hormone, or hypothermia. Some investigators suggest that I_{Ks} thus provides a "repolarization reserve" [40, 41, 76] when other outward repolarizing currents are reduced; *e.g.*, by remodeling of ion currents during heart failure progression [77, 78]. This role for I_{Ks} is supported by finding that specific I_{Ks} block markedly lengthens APD after "repolarization reserve" is attenuated by I_{Kr} block with 50 nM dofetilide. This observation is similar to that previously reported by us in dog ventricular muscle [39, 40].

The lack of effect of I_{Ks} on normal APD now found in human ventricular tissue in the absence of sympathetic stimulation and previously reported in dog [39, 40] and rabbit [54] occurs because I_{Ks} is relatively small compared to I_{Kr} over the range of voltages encountered during the time course of normal ventricular action potentials, and its activation is slow

compared to that of I_{Kr} in these species. Indeed we have shown that in normal human ventricular myocytes I_{Kr} in the absence of a sympathetic agonist activates rapidly (τ=31.0±7.4 ms, n=6) during depolarizations to positive potentials (+30 mV) but deactivates slowly at –40 mV (τ_1=599.9±53.9 ms, τ_2=6792.2±875.6 ms, n=8), whereas I_{Ks} activates slowly at positive potentials (τ=1005 ± 202.9 ms, n=7, at+30 mV) and deactivates comparatively rapidly (τ=132.4± 29.8 ms, n=7, at –40 mV) with respect to diastolic intervals (300 to 700 ms) associated with physiological heart rates [47, 52]..

These kinetics for I_{Ks} and I_{Kr} activation and deactivation indicate that when APD is abnormally long, or repolarization reserve is attenuated and sympathetic activation is increased, I_{Ks} block should be expected to substantially lengthen APD more while having little effect of normal APDs in the absence of sympathetic stimulation. Expectations borne out when either 10 μM chromanol 293B, 500 nM HMR-1556 or 100 nM L-735,821 was applied to normal dog ventricular muscle after APD was initially increased by exposure to E-4031 and veratrine [39], or by application of 100 nM isoproterenol, which enhanced ß-receptor activation and levels of intracellular cAMP [56].

The findings reported in this study suggest that antiarrhythmic drugs that selectively block I_{Ks} are unlikely to affect ventricular arrhythmias in the absence of sympathetic neural stimulation. However, it must be recognized that sympathetic tone is continuosly fluctuating in the *in situ* human heart, and many believe the selective I_{Ks} block in combination with beta-adrenoceptor blockade should have antiarrhythmic benefit, citing the clinical antiarrhythmic effectiveness of amiodarone, which has both I_{Ks} and beta-adrenoceptor blocking properties during chronic administration [79]. Nonetheless, another little explored antiarrhythmic strategy might be to increase, rather than to block, I_{Ks}. If I_{Ks} were increased (either pharmacologically or genetically), arrhythmia risk might be expected to be lowered; certainly, such an antiarrhythmic intervention would benefit patients with inherited or acquired LQT. Therapeutic increases in I_{Ks} would increase “repolarization reserve” and possibly reduce the risk of sudden cardiac death during progression of heart failure where I_{Kr} and I_{to} expressions are downregulated [77, 78, 80, 81].

5.5. Contribution and relative role of the rapid delayed and inward rectifier potassium channels (I_{Kr} and I_{K1}) in human, dog and rabbit ventricular repolarization

According to the main findings of this study the current density of I_{K1} is considerably less in human than in the dog and rabbit ventricle, while I_{Kr} density is about the same in the

human and dog. Due to voltage dependent activation properties of these two currents not only I_{Kr} but I_{K1} also plays an important role in initiating final ventricular repolarization. Pharmacological inhibition of I_{K1} elicits minor changes in the ventricle, but prolongs ventricular repolarization in the dog and rabbit. Block of I_{Kr} evokes modest prolongation of repolarization in the dog but largely or markedly lengthens it in the rabbit and human, respectively, suggesting an important role of I_{K1} as part of the repolarization in both species, but I_{K1} contributes more to normal repolarization in the dog and rabbit than in humans.

Figure 16 present the possible role and relation of the I_{Kr} and I_{K1} currents in the ventricular AP in the three studied species. I_{Kr} is similar in dog and human, but somewhat larger in rabbit, while I_{K1} is small in human and is larger in dog and even more larger in rabbit. This is the reason why selective I_{Kr} block (by 1 µM E-4031) produced a large APD lengthening in human, while only approximatively 50% less APD prolongation in dog. In rabbit the I_{Kr} is even larger than in dog and human, but because the I_{K1} is also higher, the selective I_{Kr} block produce a lengthening effect with a value between human and dog.

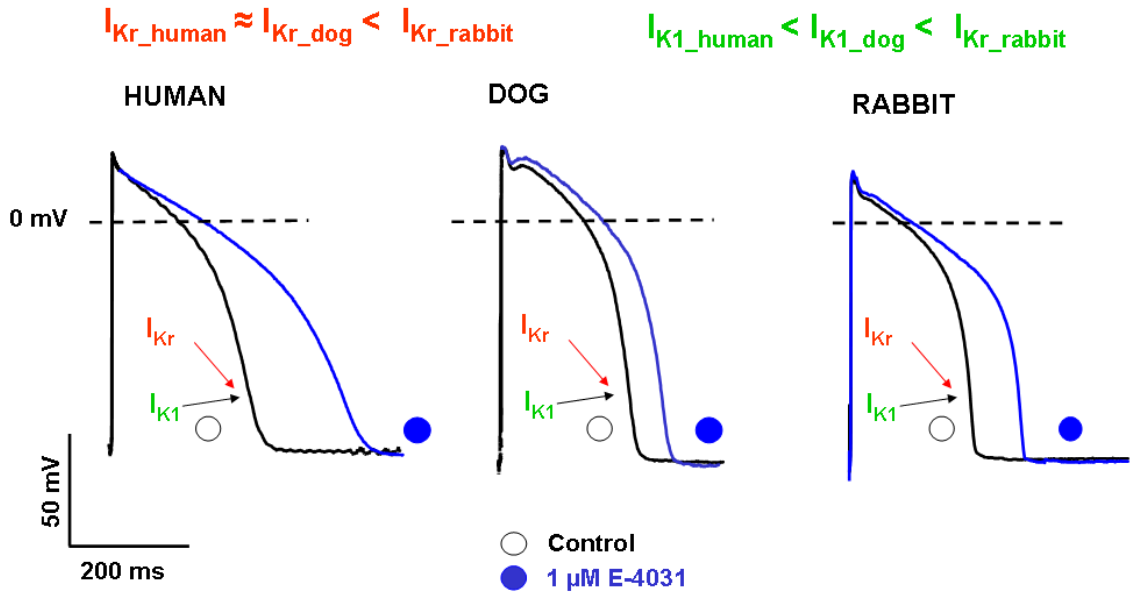

Figure 16. Relative contribution and role of the rapid delayed and inward rectifier potassium channels to human, dog and rabbit ventricular repolarization

The results showing that I_{Kr} had similar magnitude in human and dog ventricular muscle but its pharmacological depression resulted considerably more prolongation of repolarization in the human represent an interesting finding. One can speculate that in myocytes which lack strong I_{K1} like those of the human ventricle the pharmacological

inhibition of I_{Kr} or the decreased level of expression of functionally intact HERG channels due to disease caused downregulation [78] or loss of function mutation [82] would cause considerably more action potential duration prolongation than in those expected from dog studies in which strong I_{K1} is expressed. This would also help to explain the species differences between dog and human in response to I_{Kr} blocking drugs. Based on this observation and speculation it can be predicted that although the properties of the individual potassium channels in dog resembles well those of the human [39, 47, 52, 83], the drug response between the two species are not necessarily as similar as one may expect. In other words, based on experiments in the dog with drugs potentially blocking I_{Kr}/HERG channels one can underestimate the expected degree of repolarization lengthening in human. Therefore using canine preparations in certain types of safety pharmacology studies aiming to predict the possible QTc lengthening side effects of various non-cardiac drugs has no particular advantage over other animal models like the commonly used rabbit models. Their holds even if one considers the more similarity of the behaviours of the individual channels [54], and the better correlation of heart rate between human and large than small mammals.

It is also interesting to consider that I_{K1} block or downregulation/mutation would not necessarily lead to significant or large degree of QTc prolongation in human unlike in the dog, but the reduction of the repolarization reserve [40, 41, 83, 84] can be expected. Therefore, the I_{K1} (Kir2.x) channel defect or malfunction can go easily unnoticed in human. However, if decreased function or block of any other potassium channel is additionally associated, this can lead to unpredicted excessive repolarization lengthening and life threatening *torsade de pointes* arrhythmias.

It is worth noting that the results of the experiments published recently regarding the expression of biological pacemakers in dog heart [85] seem promising in the light of our present findings, since the relatively less I_{K1} in human compared to dog makes the extrapolations from the dog results to human *from this point of view* satisfactory. In human the less abundant I_{K1} current would not likely to blunt out or depress excessively the pacemaker activity of the experimentally expressed I_f channels [86], and it may therefore increase their safety to fire properly.

Possible limitations

The present work has several limitations, which should be considered. Firstly, we investigated the contribution of only two major potassium currents. The repolarization however, is a complex process involving several other transmembrane ion channels and pump

mechanisms such as I_{to}, I_{Ks}, I_{Na}, I_{Ca}, Na/K pump and Na/Ca exchanger. Therefore, for better understanding further studies are necessarily to reveal differences in both the expression and function of these channels and their contribution to repolarization between the three species.

6. Summary: conclusions and potential significance

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

1. The effect of SEA-0400, a newly developed NCX inhibitor devoid of I_{Ca} blocking property, on the formation of EAD and DAD in the dog ventricular muscle and Purkinje fibers was investigated. Evidence has been obtained for the NCX inhibitory activity of SEA-0400 and its potency to suppress elementary arrhythmogenic phenomena, such as EAD and DAD. Considering the pros and contras, further research is needed with both *in vitro* and *in vivo* methods to elucidate the potential therapeutic targets and, in a wider sense, the possible beneficial effect of specific NCX inhibition.

2. We synthesized and studied a series of compounds combining the hydroxy-benzopyran ring of vitamin E with the methylsulfonyl-aminophenyl moiety of class III antiarrhythmic drugs. These drugs were the piperazine analogues (5a-5e) and methylamino analogues (19a and 19b). Among piperazine derivatives, compounds 5c and 5e suppressed reperfusion tachycardia, while compound 5e reduced premature beats and MDA content, combining antiarrhythmic and antioxidant properties. Methylamino derivative 19a exhibited antioxidant activity and reduced premature beats, and induce a fast recovery of the heart during reperfusion. Compounds 5c, 19a and 19b facilitated the recovery of QRS and QT intervals during reperfusion, to the normal values. The cardioprotective compounds 5c-5e and 19a-19b do not induce excessive lengthening of the action potential, exhibiting moderate class III antiarrhythmic actions.

3. We studied the cellular electrophysiological effects of SZV-123 a new amiodarone-like antiarrhythmic compound that combine Class IB+III actions, in isolated dog and rabbit cardiac muscle. SZV-123 lengthened repolarization in a frequency independent manner, and decreased V_{max} only at faster rates than physiological. This property of the SZV-123 can be related to the Class I/B type sodium channel blocking effect. The frequency dependent Class I sodium channel blocking of the SZV-123 can be associated with a mexiletine-like fast restoration kinetics therefore it can be expected to decrease the conduction velocity only at

frequencies higher than physiological. It can be concluded that SZV-123 indeed possesses an amiodarone-like multichannel blocking (Class I/B + III) antiarrhythmic effects, therefore this molecule is promising for treatment of ventricular arrhythmias.

4. The role of I_{Kr} and I_{Ks} were examined in human cardiac preparations from the hearts of individuals without heart disease. I_{Ks} current in the absence of sympathetic stimulation plays no obvious role in altering action potential repolarization and QT duration at normal heart rates in human ventricular myocytes isolated. However, when human ventricular muscle repolarization reserve is attenuated and sympathetic stimulation is elevated, I_{Ks} plays an increasingly important role in limiting action potential prolongation. These findings should not be misconstrued as meaning that I_{Ks} does not play an important role in the normal heart where sympathetic stimulation is allways present and fluctuating continuously. We also believe, that I_{Ks} is vitally important in the normal heart where it prevents excessive action potential prolongation in the setting of an elevated sympathetic tone following a single long diastolic interval after the compensatory pause that follows a premature ventricular depolarization, or during bradycardia or when APD is prolonged by other means (eg, by unintentional I_{Kr} block, hypothyroidism, or serum hypokalaemia).

5. The current density of I_{K1} is considerably less in human than in the dog and rabbit ventricle, while I_{Kr} density is about the same in the three preparations. Pharmacological inhibition of I_{K1} elicits minor changes in the human ventricle, but prolongs ventricular repolarization in the dog and rabbit. Inhibition of I_{Kr} evokes modest prolongation of repolarization in the dog ventricle, but largely or markedly lengthens it in the rabbit and human, respectively, suggesting an important role of I_{K1} as part of the repolarization in both species but I_{K1} contributes more to normal repolarization in the dog and rabbit than in humans. In other words based on experiments in the dog with drugs potentially blocking I_{Kr}/HERG channels one can underestimate the expected degree of repolarization lengthening in human. Therefore using dog in certain types of safety pharmacology studies predicting the possible QTc lengthening side effects of various non cardiac drugs has no particular advantage over other animal models like the commonly used rabbit models even if one considers the more similarity the behaviours of the individual channels the better correlation of heart rate between human and large than small mammals.

7. References

1. Volders PGA, Vos MA, Szabo B, Sipido KR, Marieke de Groot SH, Gorgels APM, Wellens HJJ, Lazzara R. Progress in the understanding of cardiac early afterdepolarizations and torsades de pointes: time to revise current concepts. *Cardiovasc Res* 2000; 46: 376-392.
2. Pogwizd SM, Schlotthauer K, Li L, Yuan W, Bers DM. Arrhythmogenesis and contractile dysfunction in heart failure. Roles of sodium-calcium exchange, inward rectifier potassium current, and residual β-adrenergic responsiveness. *Circ Res* 2001; 88: 1159-1167.
3. Singh BN, Vaughan Williams EM. A third class of anti-arrhythmic action. Effect on atrial and ventricular intracellular potentials, and other pharmacological actions on cardiac muscle, of MJ 1999 and AH 3474. *Br J Pharmacol* 1970; 39: 675-687.
4. Singh B.N., A fourth class of anti-dysrhythmic action? Effect of verapamil on ouabain toxicity, on atrial and ventricular intracellular potentials, and on other features of cardiac function. *Cardiovasc Res* 1972; 6: 109-119.
5. Nattel S., Singh B. N. Evolution, mechanisms, and classification of antiarrhythmic drugs: focus on class III actions. *Am J Cardiol* 1999; 84: 11R–19R.
6. The CAST Investigators. Preliminary report: effect of encainide and flecainide on mortality in randomized trial arrhythmia suppression after myocardial infarction. *N Engl J Med* 1989; 321: 406-412.
7. Pinney SP, Koller BS, Frany MR, Woosley RL. Terfenadine increases the QT interval in isolated guinea pig heart. *J Cardiovasc Pharmacol* 1995; 25: 30-40.
8. Gintant GA, Limberis JT,McDermott JS, Wegner CD, Cox BF. The canine Purkinje fiber: an in vitro model system for acquired long QT syndrome and drug-induced arrhythmogenesis. *J Cardiovasc Pharmacol* 2001; 37: 607-618.
9. Antzelevitch C, Sun ZQ, Zhang ZQ, Yan GX. Cellular and ionic mechanisms underlying erythromycin-induced long QT intervals and torsade de pointes. *J Am Coll Cardiol* 1996; 28: 1836-1848.
10. Drici MD, Barhanin J. Cardiac K^+ channels and drug-acquired long QT syndrome. *Therapie,* 2000; 55: 185-193.
11. Rampe D, Murawsky MK. & MURAWSKY, M.K. Blockade of the human cardiac K^+ channel Kv1.5 by the antibiotic erythromycin. *Naunyn-Schmiedebergs Archiv Pharmacol* 1997; 355: 743-750.
12. Bers DM. Calcium fluxes involved in control of cardiac myocyte contraction. *Circ Res* 2000; 87: 275-281.
13. Bers D.M. Cardiac excitation-contraction coupling. *Nature* 2002; 415: 198-205.
14. Lytton J. Na^+/Ca^{2+} exchangers: three mammalian gene families control Ca^{2+} transport. *Biochem J* 2007; 406:365-82.

15. Roden DM. Antiarrhythmic drugs. In: Hardman JG, Limbird LE, Molinoff PB, Ruddon RW: Goodman Gilmans *The Pharmacological Basis of Therapeutics* 1996; Chapter 35: P839-P871.

16. Pogwizd, S.M & Bers, D.M. Calcium cycling in heart failure: the arrhythmia connection. *J Cardiovasc Electrophysiol* 2002; 13: 88-91.

17. Pogwizd, S.M. Clinical potential of sodium-calcium exchanger inhibitors as antiarrhythmic agents. *Drugs* 2003; 63: 439-452.

18. Elias CL, Lukas A, Shurraw S, Scott J, Omelchenko A, Gross GJ, Hnatowich M,. Hryshko LV. Inhibition of Na^+/Ca^{2+} exchange by KB-R7943: transport mode selectivity and antiarrhythmic consequences. *Am J Physiol Heart Circ Physiol* 2001; 281: H1334-H1345.

19. Watano T, Harada Y, Harada K, Nishimura N. Effect of Na^+/Ca^{2+} exchange inhibitor, KB-R7943 on ouabain-induced arrhythmias in guinea-pigs. *Br J Pharmacol* 1999; 127: 1846-1850.

20. Magee WP, Deshmukh G, Deninno MP, Sutt JC, Chapman JG, Tracey WR. Differing cardioprotective efficacy of the Na^+/Ca^{2+} exchanger inhibitors SEA0400 and KB-R7943. *Am J Physiol Heart Circ Physiol* 2003; 284: H903-H910.

21. Tanaka H, Nishimaru K, Aikawa T, Hirayama W, Tanaka Y, Shigenobu K. Effect of SEA0400, a novel inhibitor of sodium-calcium exchanger, on myocardial ionic currents. *Br J Pharmacol* 2002; 135: 1096-1100.

22. Matsuda T, Arakawa N, Takuma K, Kishida Y, Kawasaki Y, Sakaue M, Takahashi K, Takahashi T, Suzuki T, Ota T, Hamano-Takahashi A, Onishi M, Tanaka Y, Kameo K, Baba A. SEA-0400, a novel and selective inhibitor of the Na^+-$Ca2^+$ exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. *J Pharmacol Exp Ther* 2001; 298: 249-256.

23. Iwamoto T, Kita S, and Shigekawa M. Functional analysis of Na^+/Ca^{2+} exchanger using novel drugs and genetically engineered mice. *Folia Pharmacol Jpn* 2002; 120: 91P-93P.

24. Iwamoto T, Inoue Y, Ito K, Sakaue T, Kita S, Katsuragi T. The exchanger inhibitory peptide region-dependent inhibition of Na^+/Ca^{2+} exchange by SN-6 [2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester], a novel benzyloxyphenyl derivative. *Mol Pharmacol* 2004; 66: 45-55.

25. Roy D, Talajic M, Dorian P, Connolly S, Eisenberg MJ, Green M, Kus T, Lambert J, Dubuc M, Gagné P, Nattel S, Thibault B. Amiodarone to prevent recurrence of atrial fibrillation. Canadian Trial of Atrial Fibrillation Investigators. *N Engl J Med* 2000; 342: 913-920.

26. Singh SN, Patrick J, Patrick J.Antiarrhythmic Drugs. *Curr Treat Options Cardiovasc Med* 2004; 6: 357-364.

27. Waldo AL, Camm AJ, deRuyter H, et al., for the SWORD Investigators: Effect of d-sotalol on mortality in patients with left ventricular dysfunction after recent and remote myocardial infarction. *Lancet,* 1996; 348: 7-12.

28. Brendorp B, Elming H, Jun, L, Kober L, Malik M, Jensen GB, Torp-Pedersen, C. The DIAMOND Study Group. QTc interval as a guide to select those patients with congestive heart failure and reduced left ventricular systolic function who will benefit from antiarrhythmic treatment with dofetilide. *Circulation* 2001; 103: 1422-1427.

29. Brendorp B., Pedersen O., Torp-Pedersen C., Sahebzadah N., Kober L. A benefit-risk assessment of class III antiarrhythmic agents. *Drug Saf* 2002; 25: 847–865.

30. Purerfellner H. Recent developments in cardiovascular drug therapy: treatment of atrial arrhythmias with new class III drugs and beyond. *Curr Med Chem-Cardiovasc Hematol Agents* 2004; 2: 79–91.

31. Kathofer S., Thomas D., Karle C. A. The novel antiarrhythmic drug dronedarone: comparison with amiodarone. *Cardiovasc Drug Rev* 2005; 23: 217–230.

32. Carlson M. Azimilide dihydrochloride. *Expert Rev Cardiovasc*. Ther. 2005; 3: 387–391.

33. Sager P. T. New advances in class III antiarrhythmic drug therapy. *Curr Opin Cardiol* 2000; 15: 41–53.

34. Jurkiewicz NK, Sanguinetti MC. Rate-dependent prolongation of cardiac action potentials by a methanesulfonanilide class III antiarrhythmic agent. Specific block of rapidly activating delayed rectifier K^+ current by dofetilide. *Circ Res* 1993; 71: 75-83.

35. Vos M. A. Preclinical evaluation of antiarrhythmic drugs: new drugs should be safe to be successful. *J Cardiovas. Electrophysiol* 2001; 12: 1034–1036.

36. MacKenzie I. Safety pharmacology requirements for the development of human cardiac/cardiovascular pharmaceuticals. *Drug Dev Res* 2002, 55: 73–78.

37. Koufaki M., Calogeropoulou T., Rekka E., Chryselis M., Papazafiri P., Gaitanaki C., Makriyannis A. Bifunctional agents for reperfusion arrhythmias: novel hybrid vitamin E/class I antiarrhythmics. *Bioorg Med Chem* 2003; 11: 5209–5219.

38. Nerbonne M and Kass RS. Molecular physiology of cardiac repolarization, *Physiol Rev* 2005; 85: 1205–1253.

39. Varró A, Baláti B, Iost N, Takács J, Virág L, Lathrop Da, Lengyel C, Tálosi L, Papp JGy. The role of the delayed rectifier component I_{Ks} in dog ventricular muscle and Purkinje fibre repolarization. *J Physiol* 2000; 523: 67-81.

40. Biliczki P, Virág L, Iost N, Papp JGy, Varró A. Interaction of different potassium channels in cardiac repolarization in dog ventricular preparations: role of repolarization reserve. *Br J Pharmacol* 2002; 137: 361-368.

41. Roden DM. Taking the "idio" out of "idiosyncratic": Predicting torsades de pointes. *PACE* 1998; 21: 1029-1034.

42. Wang Z, Tristani-Firouzi M, Xu Q, Lin M, Keating MT, Sanguinetti MC. Functional effects of mutations in KvLQT1 that cause long QT syndrome. *J Cardiovasc Electrophysiol* 1999; 10: 817-826.

43. Tomaselli GF, Marban E. Electrophysiological remodeling in hypertrophy and heart failure. *Cardiovasc Res* 1999; 42: 270-283.

44. Sanguinetti MC, Jurkiewicz NK. Two components of cardiac delayed rectifier K^+ current. Differential sensitivity to block by class III antiarrhythmic agents. *J Gen Physiol* 1990; 96: 195-215.

45. Gintant GA. Two components of delayed rectifier current in canine atrium and ventricle. Does I_{Ks} play a role in the reverse rate dependence of Class III agents? *Circ Res* 1996; 78: 26-37.

46. Salata JJ, Jurkiewicz NK, Jow B, Folander K, Guinosso PJ Jr, Raynor B, Swanson R, Fermini B. I_K of rabbit ventricle is composed of two currents: evidence for I_{Ks}. *Am J Physiol* 1996; 271: H2477-H2489.

47. Iost N, Virág L, Opincariu M, Szecsi J, Varro A, Papp JG. Delayed rectifier potassium current in undiseased human ventricular myocytes. *Cardiovasc Res* 1998; 40: 508-515.

48. Hondeghem LM, Snyders DJ. Class III antiarrhythmic agents have a lot of potential but a long way to go. Reduced effectiveness and dangers of reverse use dependence. *Circulation* 1990; 81: 686-690.

49. Hohnloser SH, Woosley RL. Sotalol. *New Engl J Med* 1994; 331: 31-38.

50. Roden DM, Lazzara R, Rosen M. Multiple mechanisms in the long QT syndrome: current knowledge, gaps, and future directions. *Circulation* 1996; 94: 1996-2012.

51. Li GR, Feng J, Yue L, Carrier M, Nattel S. Evidence for two components of delayed rectifier K^+ current in human ventricular myocytes. *Circ Res* 1996; 78: 689-696.

52. Virág L, Iost N, Opincariu M, Szolnoky J, Szécsi J, Bogáts G, Szenohradszky P, Varró A, Papp J.Gy. The slow component of the delayed rectifier potassium current in undiseased human ventricular myocytes. *Cardiovasc Res* 2001; 49:790-797.

53. Roden DM.Long QT syndrome: reduced repolarization reserve and the genetic link. *J Intern Med* 2006; 259: 59-69.

54. Lengyel Cs, Iost N, Virág L, Varro A, Lathrop DA, Papp JG. Pharmacological block of the slow component of the outward delayed rectifier current (I_{Ks}) fails to lengthen rabbit ventricular muscle QT_c and action potential duration. *Br J Pharmacol* 2001; 132: 101-110.

55. Stengl M, Volders PG, Thomsen MB, Spatjens RL, Sipido KR, Vos MA. Accumulation of slowly activating delayed rectifier potassium current (I_{Ks}) in canine ventricular myocytes. *J Physiol* 2003; 551: 777-786.

56. Volders, P.G., Stengl, M., van Opstal, J.M. Gerlach U, Spätjens RL, Beekman JD, Sipido KR, Vos MA. Probing the contribution of I_{Ks} to canine ventricular repolarization: key role for beta-adrenergic receptor stimulation. *Circulation* 2003; 107: 2753-2760.

57. Hund TJ, Rudy Y. Rate dependence and regulation of action potential and calcium transient in a canine cardiac ventricular cell model. *Circulation* 2004; 110: 3168-3174.

58. Birinyi P, Acsai K, Bányász T, Tóth A, Horváth B, Virág L, Szentandrássy N, Magyar, J, Varró A, Fülöp F, Nánási PP. Effects of SEA-0400 and KB-R7943 on Na^+/Ca^{2+} exchange current and L-type Ca^{2+} current in canine ventricular cardiomyocytes. *Naunyn-Schmiedebergs Archiv Pharmacol* 2005; 372: 63-70.

59. Kimura J, Watano T, Kawahara M, Sakai E, Yatabe J. Direction - independent block of bi-directional Na^+/Ca^{2+} exchange current by KB-R7943 in guinea- pig cardiac myocytes. *Br J Pharmacol* 1999; 128: 969-974.

60. Chen YJ, Chen SA, Chang MS, Lin CI. Arrhythmogenic activity of cardiac muscle in pulmonary veins of the dog: implication for the genesis of atrial fibrillation. *Cardiovasc Res* 2000; 48: 265-273.

61. Van Wagoner DR, Nerbonne JM. Molecular basis of electrical remodeling in atrial fibrillation. *J Mol Cell Cardiol* 2000; 32: 1101-1117.

62. Yue L, Feng J, Gaspo R, Li G-R, Wang Z, Nattel S. Ionic remodeling underlying action potential changes in a canine model of atrial fibrillation. *Circ Res* 1997; 81: 512-525.

63. Studer R, Reinecke H, Bilger J, Eschenhangen T, Bohm M, Hasenfuss G, Just H, Holtz J, Drexler H. Gene expression of the cardiac Na^+-Ca^{2+} exchanger in end-stage human heart failure. *Circ Res* 1994; 75: 443-453.

64. Blaauw, Y., Beier, N., Van der Voort, P., Van Hunnik, A., Schotten, U. & Allessie, M. A. Inhibitors of the Na^+/H^+ exchanger cannot prevent atrial electrical remodeling in the goat. *J Cardiovasc Electrophysiol* 2004; 15: 440-446.

65. Levi A, Brooksby P, Hancox JC. One hump or two? The triggering of calcium release from the saarcoplasmic reticulum and the voltage dependence of contraction in mammalian cardiac muscle. *Cardiovas Res* 1993; 27: 1743-1757.

66. Bassani RA, Bassani JW, Bers DM. Relaxation in ferret ventricular myocytes: role of the sarcolemmal Ca-ATPase. *Pflügers Arch* 1995; 430: 573-578.

67. Choi HS, Eisner DA. The effects of inhibition of the sarlocemmal Ca-ATPase on systolic calcium fluxes and intracellular calcium concentration in rat ventricular myocytes. *Pflügers Arch* 1999; 437: 966-971.

68. Campbell TJ. Resting and rate-dependent depression of maximum rate of depolarisation (V_{max}) in guinea pig ventricular action potentials by mexiletine, disopyramide, and encainide. *J Cardiovasc Pharmacol* 1983; 5:291-296.

69. Bosch Rf, Gaspo R, Busch AE, Lang HJ, Nattel S. Effects of the chromanol 293B, a selective blocker of the slow component of the delayed rectifier K^+ current, on repolarization in human and guinea pig ventricular myocytes. *Cardiovasc Res* 1998; 38: 441-450.

70. Schreieck J, Wang Y, Gjini V, Korth M, Zrenner B, Schomig A, Schmitt C. Differential effect of beta-adrenergic stimulation on the frequency-dependent electrophysiologic actions of the new class III antiarrhytmics dofetilide, ambasilide, and chromanol 293B. *J Cardiovasc Electrophysiol* 1997; 8: 1420-1430.

71. Sun ZQ, Thomas G, Antzelevitch C. Role of the delayed rectifier component I_{Ks} in cardiac repolarization. – Reply. *J Cardiovasc Electrophysiol* 2001;10: 1205-1206.

72. Sun ZQ, Thomas GP, Antzelevitch C. Chromanol 293B inhibits slowly activating delayed rectifier and transient outward currents in canine left ventricular myocytes. *J Cardiovasc Electrophysiol* 2001; 12: 472-478.

73. Salata JJ, Jurkiewicz NK, Sanguinetti MC, Siegl PK, Claremon DA, Remy DC, Elliot JM, Libby BE . The novel Class III antiarrhythmic agent, L-735821 is a potent and selective blocker of I_{Ks} in guinea pig ventricular myocytes (abstract). *Circulation* 1996; 94: 3095.

74. Thomas GP, Gerlach U, Antzelevitch C. HMR 1556, a potent and selective blocker of slowly activating delayed rectifier potassium current. *J Cardiovasc Pharmacol* 2003; 41: 140-147.

75. Han W, Wang Z, Nattel S. Slow delayed rectifier current and repolarization in canine cardiac Purkinje cells. *Am J Physiol* 2001; 280: H1075-H1080.

76. Silva, J. & Rudy, Y. Subunit interaction determines I_{Ks} participation in cardiac repolarization and repolarization reserve. *Circulation* 2005; 112: 1384-1391.

77. Kaab S, Nabauer M. Diversity of ion channel expression in health and disease. *Eur Heart J Suppl* 2001; K: K31-K40.

78. Volders PG, Sipido KR, Vos MA, Spatjens RL, Leunissen JD, Carmeliet E, Wellens HJ. Downregulation of delayed rectifier K^+ currents in dogs with chronic complete atrioventricular block and acquired torsades de pointes. *Circulation* 1999; 100: 2455-2461.

79. Kamiya K, Nishiyama A, Yasui K, Hojo M, Sanguinetti MC, Kodama I. Short- and long-term effects of amiodarone on the two components of cardiac delayed rectifier $K(^+)$ current. *Circulation* 2001; 103: 1317-1324.

80. Xu XP, Salata JJ, Wang JX, Wu Y, Yan GX, Liu TX, Marinchak RA, Kowey PR. Increasing I(Ks) corrects abnormal repolarization in rabbit models of acquired LQT2 and ventricular hypertrophy. *Am J Physiol* 2002; 283: H664-H670.

81. Salata JJ, Jurkiewicz NK, Wang JX, Evans BE, Orme HT, Sanguinetti MC . A novel benzodiazepine that activates cardiac slow delayed rectifier K+ currents. *Mol Pharmacol* 1998; 54: 220-230.

82. Ehrlich JR, Pourrier M, Weerapura M, Ethier N, Marmabachi AM, Hébert TE, Nattel S. KvLQT1 modulates the distribution and biophysical properties of HERG. A novel alpha-subunit interaction between delayed rectifier currents. *J Biol Chem* 2004; 279: 1233-1241.

83. Jost N, Virág L., Baláti B, Lengyel C, Németh M, Bitay M., Bogáts G., Varró A., Papp JGy. The comparative study of the rapid (I_{Kr}) and slow (I_{Ks}) delayed rectifier potassium currents in undiseased human, dog, rabbit and guinea pig cardiac ventricular preparations. *Cardiol Hung* 2004; 34, Suppl. E: E75-E84.

84. Roden DM. Long QT syndrome: reduced repolarization reserve and the genetik link. *J Internal Medicine* 2006; 259: 59-69.

85 Qu J, Plotnikov AN, Danilo P Jr, Shlapakova I, Cohen IS, Robinson RB, Rosen MR. Expression and function of a biological pacemaker in canine heart. *Circulation* 2003; 107: 1106-1109.

86. Robinson RB, Brink PR, Cohen IS, Rosen MR. I(f) and the biological pacemaker. *Pharmacol Res* 2006; 53: 407-415.

8. Acknowledgments

I am very grateful to Professor András Varró, *MD, PhD, DS*c, for his continuous support and for providing me the opportunity to work as PhD student at the Department of Pharmacology & Pharmacotherapy, University of Szeged chaired by him. I always enjoyed his optimistic attitude to the scientific problems, and I benefited much from his zest of life and purposefulness.

I am especially thankful to associate professor Dr. Norbert Jost, my PhD supervisor for his personal guidance and for introducing me to such a challenging research area as the cardiovascular cellular electrophysiology.

I wish to thank to my colleagues, Dr. László Virág, Dr. András Tóth, Dr. Péter Biliczki, Dr. Viktória Szűts, Dr. Károy Acsai, Attila Farkas for the kindness, as their continuously guided and helped me during my PhD studies.

I thank Mrs. Zsuzsa Molnár, Ms. Éva Szabadi, Mr. Gyula Horváth and Mr. Gábor Girst for their helpful technical assistance.

Finally, I wish to thank the enthusiastic support of my family.

9. Annex

Publications related to the subject of the Thesis