ELECTROPHYSIOLOGICAL INVESTIGATION OF ANTIARRHYTHMIC COMPOUNDS: MECHANISMS AND THE SIGNIFICANCE OF REPOLARIZATION IN THE PROARRHYTHMIC EFFECTS

Ph.D. Thesis

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1. List of publications related to the subject of the Thesis

Full length papers

I Péter Biliczki, László Virág, Norbert Jost, Julius Gy. Papp, András Varró. Interaction of different potassium channels in cardiac repolarization in dog ventricular preparations: role of repolarization reserve. *British Journal of Pharmacology* 2002; 137:361-368. **IF(2002): 3.45**

II. Péter Biliczki, Károly Acsai, László Virág, Norbert Jost, András Biliczki ,Julius Gy. Papp, András Varró. Electrophysiological effect of terikalant in dog cardiac muscle. *European Journal of Pharmacology* 2005; 510(3): 161-166. **IF(2003): 2.352**

III András Varró, Péter Biliczki, Norbert Jost, László Virág, Ottó Hála, Péter Kovács, Péter Mátyus, Julius Gy. Papp. Theoretical Possibilities for the Development of Novel Antiarrhythmic Drugs. *Current Medicinal Chemistry* 2004; 11: 1-11. **IF(2003): 4.409**

IV. Norbert Jost, László Virág, Miklós Bitay, János Takács, Csaba Lengyel, Péter Biliczki, Gábor Bogáts, David A. Lathrop, Julius Gy. Papp, András Varró. Restricting excessive cardiac action potential and QT prolongation: a vital role for Iks in human ventricular muscle. Submitted to Circulation.

V. László Virág, Norbert Jost, Péter Biliczki, Julius Gy. Papp, András Varró. ORM-10103, a Novel Inhibitor of Sodium/Calcium Exchanger Devoid of Calcium and Sodium Channel Blocking Properties, Decreases Early and Delayed Afterdepolarizations in Canine Heart. Manuscript in preparation

Quotable abstracts

VI. Biliczki P, Virág L, Jost N, Papp J.Gy, Varró A. A repolarizációban részt vevő különböző kálium csatornák kölcsönhatásának vizsgálata kutya kamrai izmon: a „repolarizációs tartalék" szerepe. *Magyar Kardiológusok Társasága Tudományos Ülése, Balatonfüred, 2002. április 30-május 3. Cardiol Hung* 2002; 32 Suppl: p36.

VII. Biliczki P, Virág L, Jost N, Papp JGy, Varró A. Interaction of different potassium channels in cardiac repolarization in dog ventricular preparations: role of

repolarization reserve. *The First Multilateral Conference of the Physiologists from Central Europe, February 5-8, 2002. Piestany, Slovak Republik. Abstract Book* 2002; 31.

VIII. Biliczki P, Virág L, Jost N, Papp JGy, Varró A. Interaction of different potassium channels in dog ventricular repolarization: role of the repolarization reserve. *22nd European Section Meeting of the International Society for Heart Research, Szeged. J Mol Cell Cardiol* 2002; 34: A10.

IX. Varró A, Biliczki P, Virág L, Jost N, Papp JGy. Interaction of different potassium channels in dog ventriuclar repolarization: role of repolarization reserve. *4th International Congress of Pathopyhsiology. Acta Physiol Hung* 2002; 89: 116.

2. Introduction

Cardiovascular diseases, and in particular, cardiac arrhythmias, such as ventricular fibrillation have a leading role in mortality in the developed countries. The most serious ventricular arrhythmia—ventricular fibrillation—causes the death of more than 3 000 000 people all over the world and 300-350 000 people in the USA and Europe annually, which statistically means that one person dies in every minute on each continent. In Hungary exact data are not available, but according to calculations there are 25-26 000 sudden cardiac death cases annually, meaning 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 social and economic outcomes, since it is the head of the family, a seemingly healthy man, who dies tragically in most of the cases. 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. 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.

Concepts regarding the treatment of cardiac arrhythmias changed significantly in the past decade, owing to the revolutionary developing electrophysiological methodes (patch-clamp, molecular biology). The Cardiac Arrhythmia Supression Trial (CAST) [1] 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. Consequently, since the CAST, the interest of drug development and treatment of ventricular tachycardia (VT) and atrial fibrillation (AF) has been shifted toward those agents that prevent and terminate reentrant arrhythmias by prolonging the action potential duration (APD) and effective refractory periode (ERP), resulting in an increase in arrhythmia wavelength and a block development within the reentrant circuit.

Class III antiarrhythmic action, i.e.lengthening of cardiac action potential duration (APD) [2] and prolongation of the repolarization, is usually caused by blockade of one or more potassium channels. Sodium channels are not affected, thus conduction velocity remains unchanged. A great number of non-cardiac drugs do result in lengthening of repolarization in both ventricular muscle cells and Purkinje fibers by using a similar mode of action [3].

The repolarization of the cellular membrane is achieved mainly by the rapid delayed rectifier potassium current (I_{K1}). In most species, including humans, it is made of a rapid (I_{Kr}) and a slow (I_{Ks}) component [4]. These two components are different in terms of drug sensitivity as well as in the characteristics and kinetics of their voltage-current relationships. Selective blockers of I_{Kr} (d-sotalol, ibutilide, dofetilide) significantly lengthen the APD in heart muscle cells, and their antiarrhythmic effect has been proven in human as well. However, the Suppression With Oral D-sotalol (SWORD) trial [5] has showed that d-sotalol increased the mortality (presumably due to Torsade de Pointes arrhythmia) in postinfarction patients with ventricular arrhythmia.

This adverse effect of d-sotalol on mortality is most likely due to its bradycardia-dependent proarrhythmic („torsadogenic") action and most other pure Class III compounds might act in a similar harmful way. The outcome of the SWORD study has seriously affected the

development of selective Class III compounds, namely, research on several compounds belonging to this group has been discontinued [6].

Most drugs with pure Class III action prolong APD more at slow rates and produce little or no change at fast ones. This phenomenon, termed reverse rate-dependency [7] is particularly evident in M cells and Purkinje fibers [8]. The reverse rate-dependent nature of APD lengthening effect of most pure Class III drugs seriously limits the antiarrhythmic efficacy by compromising their ability to prolong APD and refractoriness when most needed, namely during tachycardia. In addition, it contributes importantly to the proarrhythmia caused by Class III agents because of the excessive prolongation of repolarization. Emphasis was therefore shifted toward developing compounds with a multifaceted ("hybrid") pharmacological profile [9,10] with multiple molecular targets suggested by the "Sicilian gambit" [11]. Many investigators are now considering that application of drugs with multiple actions like amiodarone would represent a possible effective way to treat both ventricular and supraventricular arrhythmias [12]. Within the group of these drugs having multiple molecular targets, RP 58866, and its enantiomer, terikalant were first considered as selective blockers of the inward rectifier potassium current (I_{K1}) [13]. These compounds exhibited efficacy in experimental arrhythmias [14], suggesting that block of I_{K1} may be a useful antiarrhythmic mechanism with lower incidence of the torsade de pointes ventricular tachycardias [15]. The in vitro electrophysiological effects of terikalant, this new investigational antiarrhythmic drug with largely unknown cellular mode of action is being extensively studied. I investigated the cellular mechanisms of action of terikalant in canine isolated ventricular muscle and Purkinje fibers, by applying the standard microelectrode technique.

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. 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, since the other potassium currents may provide sufficient repolarizing capacity, which can be considered as a 'repolarization reserve'. However, in situations where the density of one or more types of potassium channels is decreased by inheritance or remodelling [16,17] 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.

Recently, experiments carried out in our lab suggested that selective I_{Ks} block only minimally lengthens repolarization in normal dog ventricular muscle. However, when the 'repolarization reserve' is attenuated by E-4031 or veratrine [18], I_{Ks} block substantially delays repolarization. One of the goals of my PhD project was to shed more light on the possible role of the 'repolarization reserve' and the interaction of different potassium channels in cardiac repolarization using the dog ventricular muscle as a model system.

The rapid component of the delayed rectifier potassium current (I_{Kr}) has been identified in several mammalian species [19,20,21], including human [22,23,24,25]. 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 Torsade de Pointes [26,27]. Mutations in ion channel genes, including HERG and KCNE2, that suppress I_{Kr} result in a specific form of the inherited long QT syndrome, LQT2, are also associated with rhythm disorders and an increased incidence of Sudden Cardiac Death (SCD) [28]. As such, I_{Kr} plays a major role in action potential repolarization in health and in specific cases of arrhythmogenesis [29].

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 [19,20,21], including humans [24,29] 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 [28]. In contrast, 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 [30,31] over a wide range of physiologic pacing frequencies. 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. Our previously reported findings have now been confirmed by those of other investigators [31] and supported by computer simulations that suggest that I_{Ks} does not play a role in adaptations of APD to changes in heart rate [32]. However, the role of I_{Ks} in human ventricular muscle remains

controversial; although, our preliminary characterization of I_{Kr} [25] and I_{Ks} [29] in isolated human ventricular myocytes suggests that these currents behave much the same as they do in isolated dog [19] and rabbit [30,20] ventricular myocytes. The purpose of the present study, therefore, was to confirm our initial findings while better elucidating the role of I_{Ks} in normal human ventricular muscle action potential repolarization.

Prolongation of cardiac repolarization is an important mode of action of pure Class III drugs (i.e. those that block I_{Kr} selectively). These compounds should be applied with caution, since they result in increased mortality due to several reasons. First, they increase the inhomogenity of repolarization and consequently that of the refractoriness. Second, the reverse use-dependent effect of these drugs (i.e. that they cause greater prolongation of the APD at slow versus rapid rates of stimulations) is also disadvantageous because at slow heart rate it may cause early afterdepolarizations (EAD) and consequently Torsade de Pointes (TdP) type ventricular arrhythmias.

In our study we investigated the rate dependent effect of the different drugs.

Another arrhythmogen factor that can result in ventricular arrhythmias occuring in myocardiac ischaemia or poisoning with digitalis is delayed afterdepolarization (DAD), which arises in heart muscle cells following Ca^{2+} overload. Reducing the incidence of these two trigger mechanismus (EAD and 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) [33]. 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 [34,35], especially in conditions where potassium conductance is decreased, such as in heart failure [36]. Therefore one may speculate that specific blockers of NCX can be potential antiarrhythmics in dysrhythmias related to Ca^{2+} overload. 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 decreases intracellular Ca^{2+} load, thereby indirectly changing the

magnitude of NCX. Recently it was found that KB-R7943, an effective inhibitor of NCX in the reverse mode but not in the forward mode [37], reduced the incidence of ischaemia and reperfusion arrhythmia induced by calcium overload [38,39]. However, KB-R7943 also inhibits the L-type calcium current [40] which makes the interpretation of its antiarrhythmic effect rather uncertain. Therefore, we investigated the effect of ORM-10103, 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 fibers, using the conventional microelectrode technique.

3. Major specific experimental aims

a) To investigate the *in vitro* electrophysiological effects of terikalant, this new investigational antiarrhythmic drug with largely unknown cellular mode of action.

b) To study the possible role and the interaction of different potassium channels in cardiac repolarization in dog ventricular muscle.

c) To elucidate the role of I_{Kr} and I_{Ks} in normal human ventricular muscle.

d) To study the effect of ORM-10103, 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 fibers.

4. Methods

4.1 Experimental animals

Mongrel dogs of either sex (body weights 8-20 kg) were used for the study. All experiments were carried out in compliance with the Guide for the Care and Use of

Laboratory Animals (U.S.A: NIH publication No 85-23, revised 1985). The protocols were approved by the Review Board of the Comittee on Animal Research of the University of Szeged (54/ 1999).

4.2 Preparations

Endocardial preparations (obtained from papillary muscles) were isolated from the right ventricle of hearts removed from anaesthetized (sodium pentobarbital 30 mg/kg iv.) mongrol dogs of either sex. Free running false tendons of Purkinje fibers 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) Tyrode's solution (flow 4-5 ml/min) warmed to 37 °C (pH 7.3 ± 0.5) and containing (in mM/l) NaCl 123, KCl 4.7, $NaHCO_3$ 20, $CaCl_2$ 1.8, $MgCl_2$ 0.8 and D-glucose 10. Preparations were oxygenated also in the tissue bath directly.

4.3 Conventional microelectrode technique

Adult mongrel dogs (8 ± 14 kg) of either sex were used. Following anaesthesia (sodium pentobarbitone, 30 mg kg-1 administered intravenously), the heart of each animal was rapidly removed through right lateral thoracotomy. The hearts were immediately rinsed in oxygenated Tyrode's solution containing (in mM): NaCl, 115; KCl, 4; $CaCl_2$, 1.8; $MgCl_2$, 1; $NaHCO_3$, 20; and glucose, 11. The pH of this solution was 7.40 ± 7.45 when gassed with 95% O_2 and 5% CO_2 at 37 °C. 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 at least 1 h to equilibrate 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, U.S.A.).

The maximum diastolic potential, action potential amplitude and action potential duration (APD) measured at 50 and 90% repolarization (APD_{50-90}) 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.

4.4 Patch-clamp measurements

Ventricular myocytes were enzymatically dissociated from hearts which were removed from mongrel dogs of either sex weighing 10-20 kg following anaesthesia (sodium pentobarbital, 30 mg kg^{-1} i.v.). 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^{-1}, Sigma) and bovine serum albumin (fraction V, fatty acid free, 2 mg ml^{-1}, Sigma) (15 min). Protease (type XIV, 0·12 mg ml^{-1}, 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. Normal Tyrode 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 $CaCl_2$ 1.0 (pH 7.2 adjusted with NaOH). 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). 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).

4.5 Whole cell configuration of the patch-clamp technique

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 served as the normal superfusate. This solution contained (mM): NaCl 144, NaH2PO4 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.

Patch-clamp micropipettes were fabricated from borosilicate glass capillaries (Clark, Reading, U.K.) using a P-97 Flaming/Brown micropipette puller (Sutter Co, Novato, CA, U.S.A.). 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 (114.4±26.2) was measured by applying a 10 mV hyperpolarizing pulse from -10 mV. 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). 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} blocker L-735,821 (100 nM, a gift from Merck-Sharpe & Dohme, West-Point, PA, U.S.A.). Membrane currents were recorded with Axopatch-1D and 200B patch-clamp amplifiers (Axon Instruments, Union

City, CA, U.S.A.) 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 MΩ 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, Axon Instruments) under software control (pClamp 6.0 and 7.0 Axon Instruments). Analyses were performed using Axon (pClamp 6.0) software after low-pass filtering at 1 kHz. All patch-clamp data were collected at 37 °C.

4.6 Human experiments

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 [41] 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.

Human tissue preparation

Action potentials were recorded in 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) using conventional microelectrode techniques. 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.

Action potential measurements in multicellular preparations:

Initially, each preparation was stimulated at a basic cycle length of 1000 ms (frequency = 1 Hz), using 2 ms long rectangular constant voltage pulses isolated from ground and delivered *via* bipolar platinum electrodes in contact with the preparation using an EMG 4767 type stimulator (Medicor Ltd, Budapest, H-1147, Hungary). One hour or more was allowed for each preparation to equilibrate while continuously superfused with Tyrode's solution warmed to 37 °C. Transmembrane potentials were recorded using a conventional glass microelectrode filled with 3 M KCl with a tip resistance of 5-20 MΩ connected to a high impedance electrometer (Bio-Logic VF102, CLAIX, F-38640, France) referenced to ground. The first derivative of transmembrane potential (dV/dt) was electronically derived using a Bio-Logic DV-140 (Claix, F-38640, France) differentiator designed and calibrated to have a linear response over the range of 10 to 1000 V/s. Amplifier outputs were digitized using an ADA 3300 analog-to-digital converter (Real Time Devices Inc, State College, PA 16804, USA) with a maximum sampling rate of 50 kHz connected to an IBM compatible personal computer. Data was stored and analyzed on the PC and also monitored on a dual beam memory oscilloscope (Tektronix 2230, Beaverton, OR 97077, USA). Resting membrane potential (RP), action potential amplitude (APA), and action potential durations (APD), measured at 50% and 90% repolarization (APD_{50} and APD_{90}), were automatically measured with software developed in our laboratory (APES, Hugo Sachs Elektronik, March-Hugstetten, D-79229, Germany). Stimulation pulses were also controlled by PC software providing constant current pulses with programmed timing and amplitudes to the preparation via a EMG 47671 type signal isolator (Medicor Ltd, Budapest, H-1147, Hungary).

In each experiment, baseline action potential characteristics were first obtained during superfusion with normal 37 °C Tyrode's solution while continuously pacing at a basic cycle length of 1000 ms, followed by a run of changing pacing cycle lengths (300, 400, 500, 700, 1000, 1500, 2000, 3000 and 5000 ms) sequentially applied for 25 beats each. This procedure allowed action potential parameters to be quickly obtained at each pacing cycle length after a "quasi steady-state" was established. Recordings were continuously monitored to confirm one-to-one activation throughout the procedure.

After baseline measurements were obtained, each preparation was superfused with Tyrode's solution containing a single test drug diluted to the proper concentration for 40 to 60 minutes before measurements were repeated at 3 min intervals in the continued presence of the test drug until less than a 5% change occurred in action potential characteristics between subsequent samples. When microelectrode impalement was lost, reimpalement was attempted. If action potential characteristics recorded with the new impalement deviated by more than 5% from the preceding ones, the experiment was terminated, and results were excluded from evaluation.

4.7 Drugs

Terikalant (RP62719, Rhone-Poulenc Rorer, France) was dissolved in dimethyl-sulphoxide (DMSO) at concentrations of 1 and 100 mM.

Chromanol 293B (gift from Aventis Pharma, Frankfurt, Germany) and dofetilide (Aventis Pharma, Frankfurt, D-65926, Germany) were dissolved in 100% DMSO to make 1 mM and 10 mM stock solutions. The stock solutions were further diluted in the tissue bath to obtain the desired final drug concentrations.

I_{Kr} blockers D-sotalol (Bristol-Myers Squibb Co., Wallingford, CT 06492, USA) and E-4031 (Institute for Drug Research Ltd., Budapest, H-1045, Hungary) were prepared daily from aqueous stock solutions (30 mM and 10 mM, respectively) to obtain the final drug concentration examined. I_{Ks} blockers chromanol 293B (Institute for Drug Research, Budapest, Hungary), HMR-1556 (Aventis Pharma) and L-735,821 (Merck-Sharpe & Dohme Co, West Point, PA 19486, 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.

4.8 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 (S.E.M.)

5. Results

5.1 Goal I: To investigate the in vitro electrophysiological effects of terikalant, this new investigational antiarrhythmic drug with largely unknown cellular mode of action.

5.1.1 Effects of terikalant on the action potential parameters in canine ventricular muscle and Purkinje fibers

The effect of terikalant on the duration of the action potential and on the maximum velocity of the action potential upstroke (V_{max}) at a cycle length of 1000 ms was investigated in concentrations of 1, 2.5, 10 and 20 μM in right ventricular papillary muscle preparations. Fig. 1A shows that terikalant concentration-dependently lengthened the action potential, which effect was more pronounced when the duration of the action potential was measured at 50 % of repolarization. In Fig. 1B the effect of terikalant on V_{max} at the same concentrations is shown. At low concentrations (1 and 2.5 μM) terikalant did not affect the V_{max}, although a small but non-significant increase was observed at 2.5 μM concentration, which was likely due to an improved microelectrode-cell connection observed in some experiments with time. However, application of terikalant at 10 and 20 μM concentration resulted in a marked and concentration-dependent decrease in V_{max}.

A detailed analysis of the action potentials recorded before and after the application of terikalant was carried out at 2.5 and 10 μM concentrations. Representative traces of the action potentials and the first derivatives of the action potential upstroke, dV/dt, recorded before and after administration of terikalant in canine ventricular muscle and Purkinje fibers are shown in Fig. 2. Terikalant, investigated at 2.5 μM concentration, significantly lengthened the action potential durations measured both at 50 and 90 % of repolarization from 190.4 ± 7.3 to 216.4 ± 8.2 ms ($n = 6$, $P < 0.05$) and from 224.7 ± 6.9 to 259.8 ± 9.6 ms ($n = 6$, $P < 0.05$), respectively, in papillary muscle without causing considerable change in the maximum diastolic potential, action potential amplitude and V_{max}. However, at 10 μM concentration, in addition to the lengthening of the action potential duration from 209.2 9.4 to 224.9 10.3 and from 253.9 ± 7.7 to 288.9 ± 7.4 ms ($n = 6$, $P < 0.05$), measured at 50 and 90 % of

repolarization, respectively, the drug also caused a significant decrease in V_{max} from 211.8 ± 14.1 to 183.3 ± 14.5 V/s (n = 6, P < 0.05). In Purkinje fiber 2.5 μM terikalant significantly lengthened the action potential duration measured at 90 % of repolarization from 241.9 ± 13.2 to 320.8 ± 16.2 ms (n = 6, P < 0.05) without affecting the action potential duration measured at 50 % of repolarization and the maximum diastolic potential. Also, V_{max} was significantly decreased by the drug from 747.4 ± 68.6 to 586.3 ± 79.2 V/s (n = 6, P < 0.05) and a slight but significant reduction in action potential amplitude from 132.6 ± 2.4 to 127.6 ± 3.4 mV was also found in Purkinje fibers in the presence of 2.5 μM terikalant (Table 1, Fig. 2C).

5.1.2 Frequency-dependent effects of terikalant in ventricular papillary muscle and Purkinje fiber

Fig. 3A and 3B show that the drug, at a concentration of 2.5 μM, lengthened the duration of the action potential measured at 90 % of repolarization in a reverse frequency-dependent manner both in papillary muscle and Purkinje fibers, with a more pronounced reverse frequency dependence observed in Purkinje fibers.

In papillary muscle, frequency-dependent effect of terikalant (2.5 and 10 μM) on the maximum upstroke velocity of the action potential (V_{max}) was also investigated. V_{max} was unaffected by the drug given in the lower (2.5 μM) concentration at any given stimulation rate (Fig. 4A). However, as shown in Fig. 4B 10 μM terikalant caused a rate-dependent depression in the maximal upstroke velocity of the action potential in this preparations, ie. decreasing V_{max} more at high than at slow stimulation rate.

5.1.3 Offset and onset kinetics of V_{max} block

Fig. 5B shows the offset kinetics of V_{max} block induced by 10 μM terikalant in papillary muscle preparations. The V_{max} values of premature beats elicited once after every 10^{th} basic beat (at a cycle length of 1000 ms) are plotted as a function of diastolic interval (interval between the 90% repolarization of the basic beat and the upstroke of the premature beat). The recovery curves illustrated in Fig. 4B show that the drug, in addition to the fast recovery (< 50 ms) which presumably reflects the drug free sodium channels, induced a slow phase of recovery of V_{max} with a time constant (τ) of 2956 ± 696 ms (n = 6).

The onset kinetics of V_{max} block induced by 10 μM terikalant was also studied in dog right ventricular papillary muscle (Fig. 5A). Preparations were continuously stimulated at a cycle length of 1000 ms. The stimulation was interrupted for 1 min, and then a train of 40 stimuli was applied with a cycle length of 500 ms (Fig. 5A). In control conditions, there was only a minor change between the first and last V_{max} values in the train. In the presence of 10 μM terikalant, however, a marked use-dependent V_{max} block developed with an onset kinetic rate constant of 0.6 ± 0.1 beat-1 (n = 6).

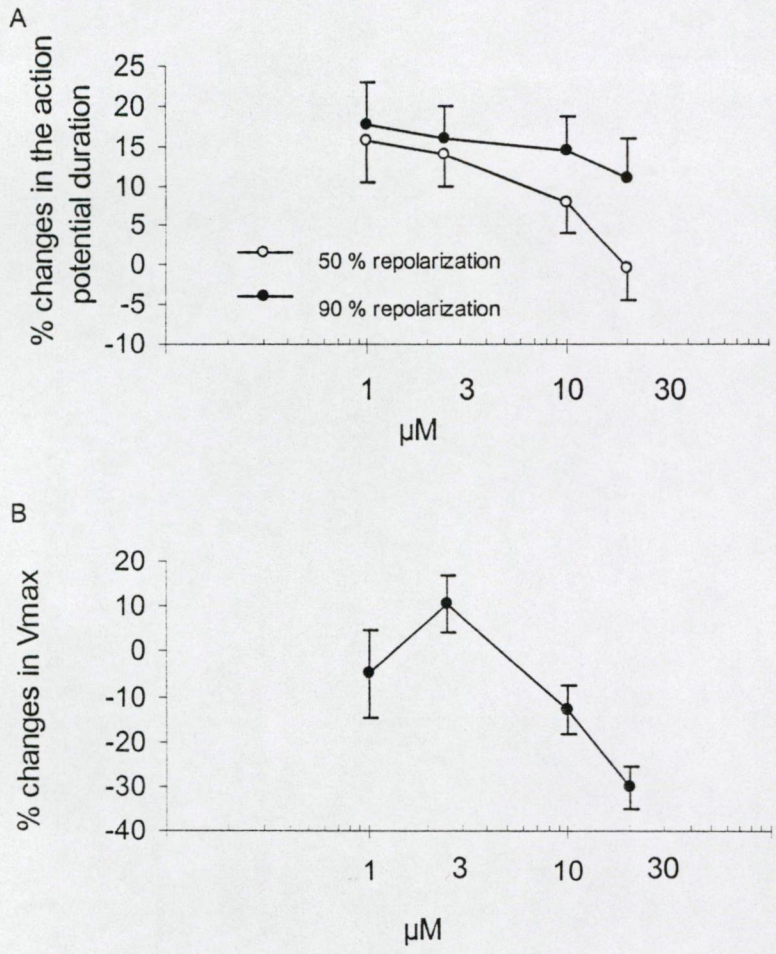


Fig. 1: Concentration-response characteristics of the effect of terikalant on duration of the action potential measured at 50 and 90 % of repolarization (A) and the maximum upstroke velocity of the action potential (V_{max}) (B) at a cycle length of 1000 ms in dog papillary muscles. Terikalant was applied at concentrations of 1, 2.5, 10 and 20 μM (n = 4-6/concentration).

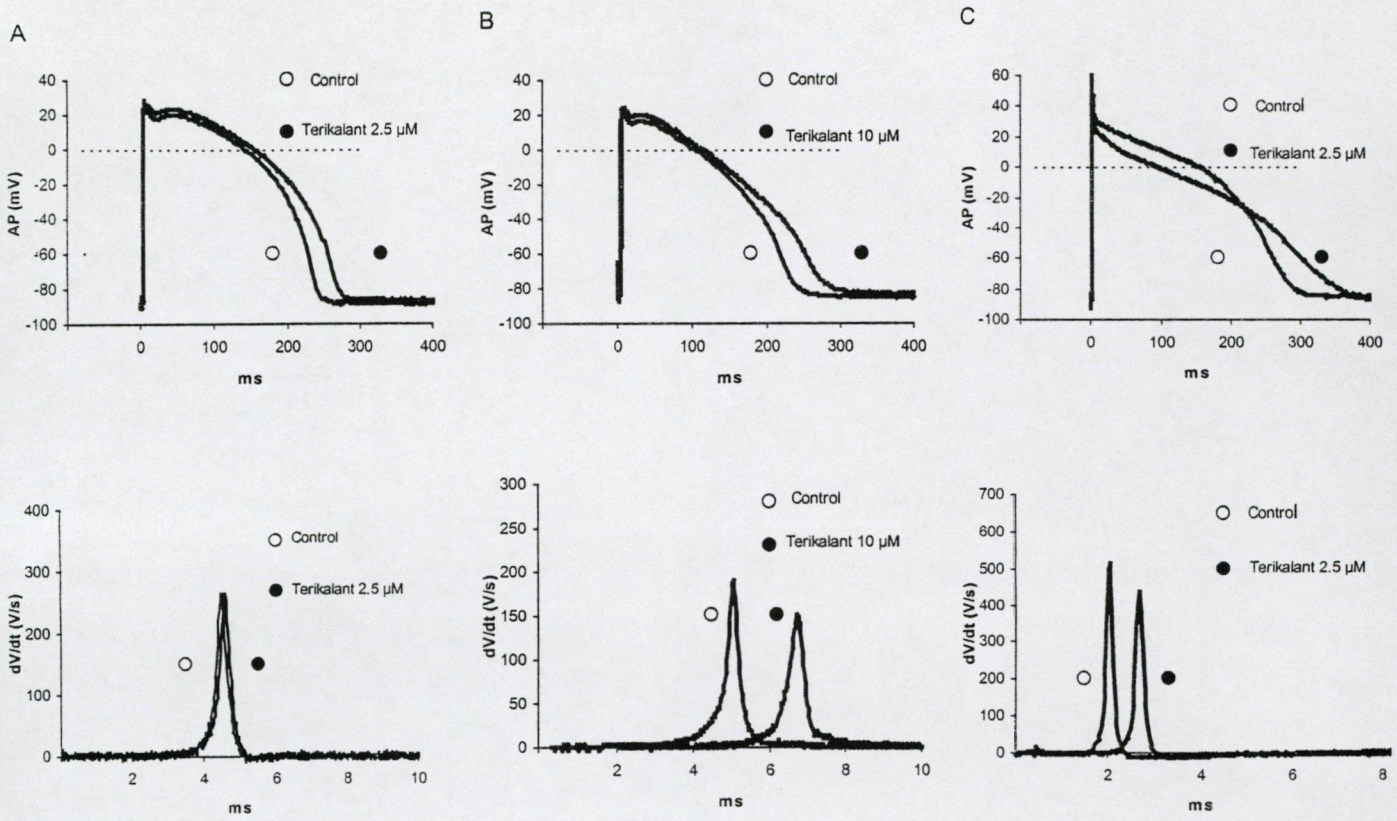


Fig. 2: Representative traces of the transmembrane action potential (top) and the first derivative of the action potential upstroke, V_{max} (bottom) recorded from right ventricular papillary muscle (A and B) and from Purkinje fiber in control conditions and in the presence of 2.5 µM (A and C) and 10 µM (B) terikalant. Traces were recorded at a cycle length of 1000 ms.

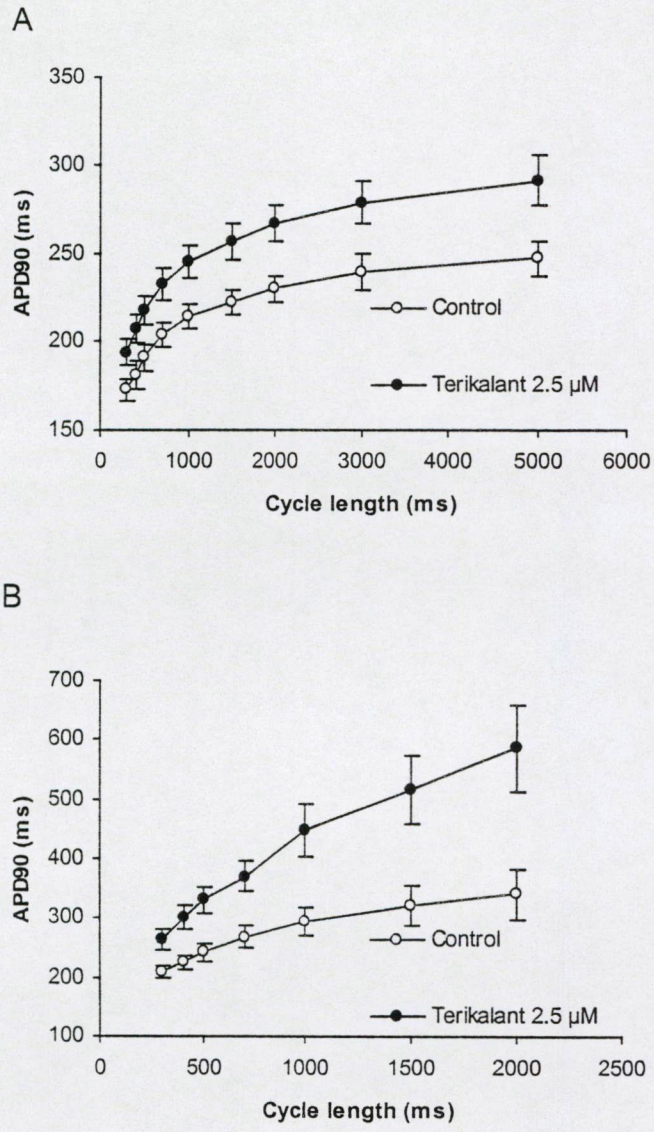
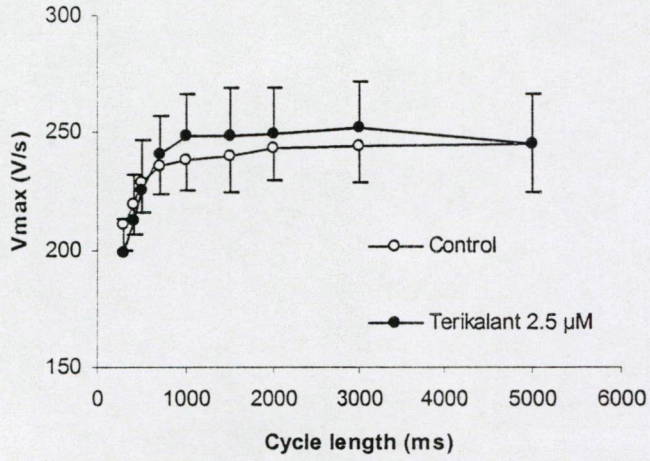


Fig. 3: Cycle length dependent effect of terikalant (2.5 µM) on the action potential duration measured at 90 % of repolarization in right ventricular papillary muscle (A) and Purkinje fiber (B) (n = 6).

A



B

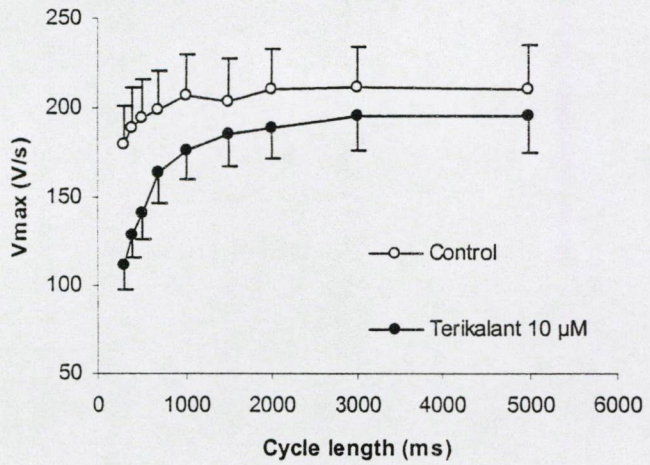


Fig. 4: Cycle length dependent effect of terikalant on the maximum upstroke velocity of the action potential (V_{max}) in right ventricular papillary muscle at the concentration of 2.5 µM (A) and 10 µM (B) (n = 6).

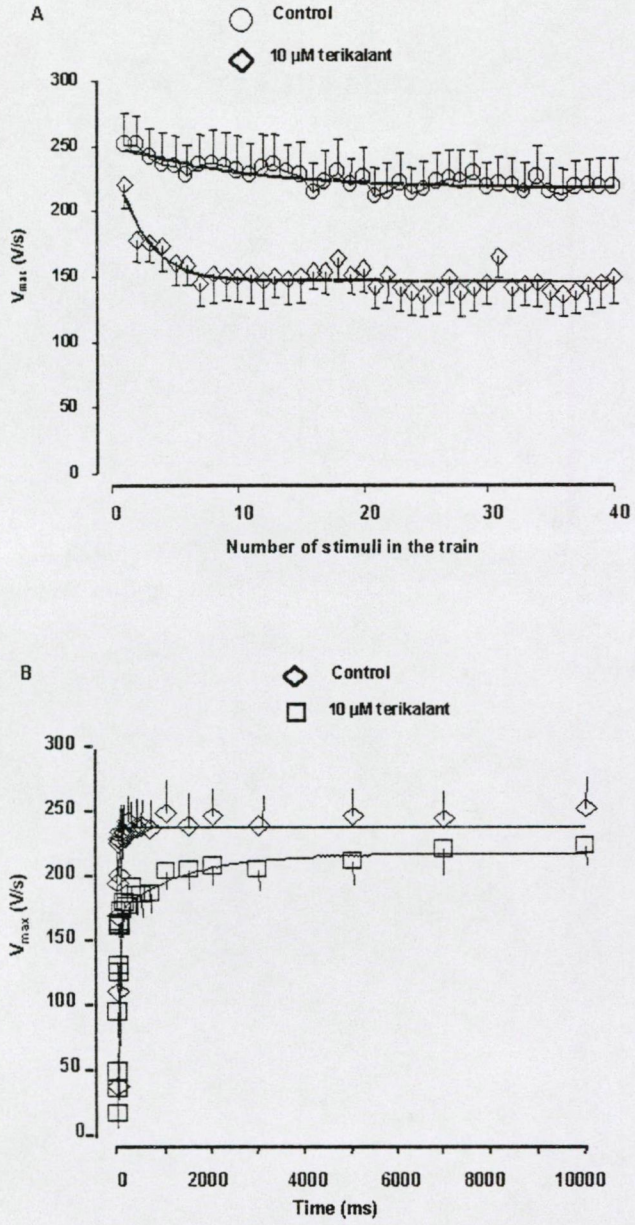


Fig. 5: Effect of 10 µM terikalant on the onset kinetics of V_{max} block (A) and on the recovery of V_{max} from inactivation (B) in canine right ventricular papillary muscle (n = 6).

5.2 *Goal II: To study the possible role and the interaction of different potassium channels in cardiac repolarization in dog ventricular muscle.*

5.2.1 *Effect of I_{Ks} and combined I_{Ks} and I_{Kr} inhibition on the APD*

Chromanol 293B is considered as an effective and relatively selective blocker of I_{Ks} (Busch et al., 1996). In our experiments we used 10 μM chromanol 293B since at higher concentrations the compound was reported also to block I_{to} (Bosch et al., 1998; Sun et al., 2001) or possibly other current(s). In order to establish the selectivity of chromanol 293B on I_{Ks} we have investigated the possible effects of chromanol 293B on various transmembrane potassium currents. In all experiments L-type calcium current (I_{Ca}) was fully blocked by addition of 1 μM nisoldipine.

I_{K1} was measured by applying 36 s long ramp pulses from -120 mV to +60 mV from the holding potential of -90 mV at the pulse frequency of 0.02 Hz. As Figure 6A shows the current traces, which represent I_{K1}, were overlapping and therefore indistinguishable from each other indicating that 10 μM chromanol 293B did not affect I_{K1}. The transient outward current (I_{to}) was elicited by 300 ms long test voltage pulses to between -20 and 50 mV from the holding potential of -90 mV with 0.33 Hz pulse frequency. The amplitude of the current was determined as the difference of the peak current at the start of the pulse and the steady-state current level measured at the end of the pulse. Figure 6B show that 10 μM chromanol 293B did not affect significantly I_{to}. The rapid delayed rectifier current (I_{Kr}) was measured by 1 s long test voltage pulses to between -10 and 50 mV with pulse frequency of 0.05 Hz. The holding potential in these experiments was -40 mV. The deactivating so-called tail current was measured as I_{Kr} after returning the voltage from the test potential to -40 mV. The amplitude of the I_{Kr} tail current was determined as the difference between the peak tail current and the holding current level at -40 mV. Figure 6C show that chromanol 293B, even at the high 100 μM concentration, exerted only minimal effect on I_{Kr}. In these experiments I_{Ks} was blocked by 100 nM L-735,821.

The slow delayed rectifier (I_{Ks}) was measured by 5 s long test voltage pulses to between −20 and +50 mV with the pulse frequency of 0.1 Hz. The holding potential in these experiments was -40 mV. I_{Kr} was completely blocked by 2 μM E-4031. The deactivating I_{Ks} tail current was measured after returning the voltage from the test potential to -40 mV. The amplitude of the I_{Ks} tail current was determined as the difference between the peak tail current

and the holding current level at -40mV. Figure 6D shows that 10 μM chromanol 293B caused a more than 50% inhibition of I_{Ks}.

These experiments suggested that chromanol 293B at 10 μM can be applied as a tool inhibiting I_{Ks} rather specifically.

Figure 7 shows that 10 μM chromanol 293B alone lengthened APD only to a small extent (<7%) in dog right ventricular papillary muscle at stimulation cycle lengths ranging from 300 to 5000 ms. However, when the same concentration of chromanol 293B was added to preparations in which I_{Kr} was previously blocked by the specific I_{Kr} blocker dofetilide (1 μM), it induced a marked and significant further prolongation of the APD (Figure 8), suggesting a strong additive effect of the two compounds.

5.2.2 Effect of I_{K1} and combined I_{K1} and I_{Kr} inhibition on the APD

It was reported that a low concentration of $BaCl_2$ inhibited I_{K1} in cardiac myocytes [42]. I_{K1} and I_{to} were measured as previously described. Since $BaCl_2$ markedly reduced I_{K1}, which appeared as a voltage independent background current when measuring I_{Kr} and I_{Ks}, the following protocols were applied to determine the effect of $BaCl_2$ on I_{Kr} and I_{Ks}. First the previously described voltage protocols were used in control condition. Then 10 μM $BaCl_2$ was added, and the voltage protocols were repeated. To distinguish between the effect of $BaCl_2$ on I_{K1} and I_{Kr}/I_{Ks}, 2 μM E-4031 or 100 nM L-735,821 were added to the tissue bath and measurements were repeated again. Subtracting current traces after application of the specific blockers for I_{Kr} or I_{Ks} in the presence of $BaCl_2$ resulted in E-4031 or L-735,821 sensitive currents, which can be considered as I_{Kr} or I_{Ks}, respectively. These E-4031 and L-735,821 sensitive currents, in the presence of $BaCl_2$, were compared to control I_{Kr} and I_{Ks} measurement to determine the possible effect of 10 μM $BaCl_2$ on I_{Kr} and I_{Ks}. As Figure 9 shows, 10 μM $BaCl_2$ did not significantly affect I_{to}, I_{Kr} and I_{Ks}. Figure 9 indicate, however, that 10 μM$BaCl_2$ inhibited I_{K1} very effectively (>70%) suggesting that at this concentration $BaCl_2$ can be used as a rather selective tool to inhibit I_{K1}.

Figure 10 shows that 10 μM $BaCl_2$ alone lengthened APD in a reverse rate dependent manner inducing APD prolongation of 9.4+2.1% at cycle length of 300 ms (n=11, $P<0.05$) and 33.0+3.3% at cycle length of 5000 ms (n=11, $P<0.05$).

When the same concentration of $BaCl_2$ was applied to preparations in which I_{Kr} was previously blocked by 1 μM dofetilide, $BaCl_2$ induced a more excessive reverse-rate dependent further prolongation of APD (Figure 11). This excessive APD lengthening at long

cycle lengths frequently (in three out of seven experiments) resulted in early afterdepolarizations (EADs) as shown in Figure 12).

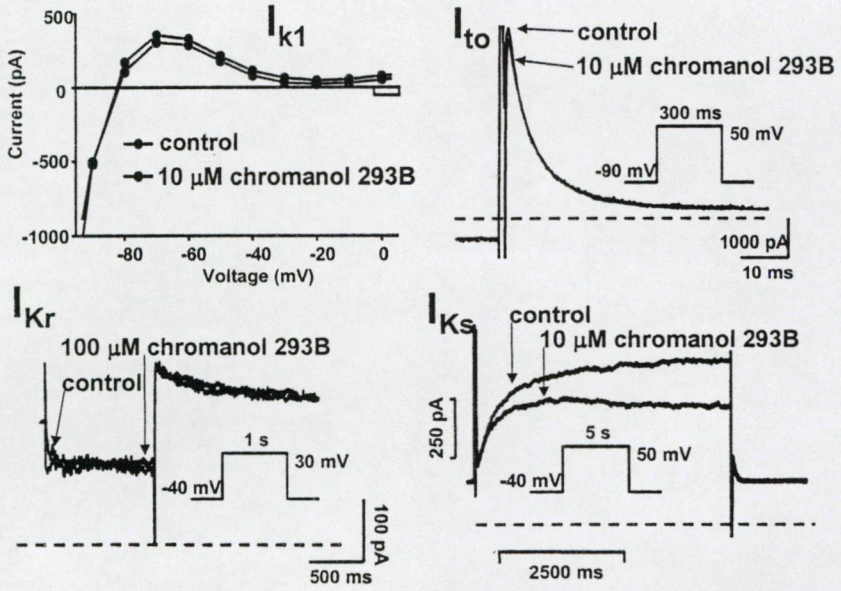


Figure 6: Effect of chromanol 293B on the inward rectifier (I_{K1}), transient outward (I_{to}), rapid delayed rectifier (I_{Kr}) and slow delayed rectifier (I_{Ks}) potassium currents in dog ventricular myocytes. When measuring I_{Kr}, L-735,821 (0.1 μM) was used to completely block I_{Ks}, and E-4031 (2 μM) was applied to block I_{Kr} when measuring I_{Ks}. The applied voltage protocols are shown in the insets and explained in the text in more detail. In all experiments I_{Ca} was fully blocked by addition of 1 μM nisoldipine. The dotted lines represent the zero current levels.

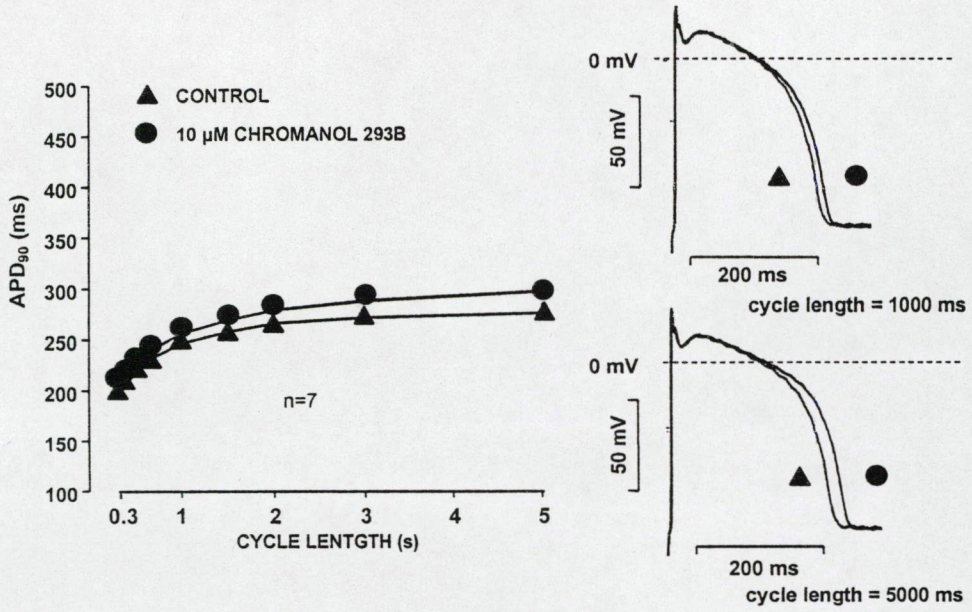


Figure 7: Frequency dependent effect of I_{Ks} block (chromanol 293B) on the action potential duration in dog right ventricular papillary muscle. Note that the error bars are often smaller than the corresponding symbols.

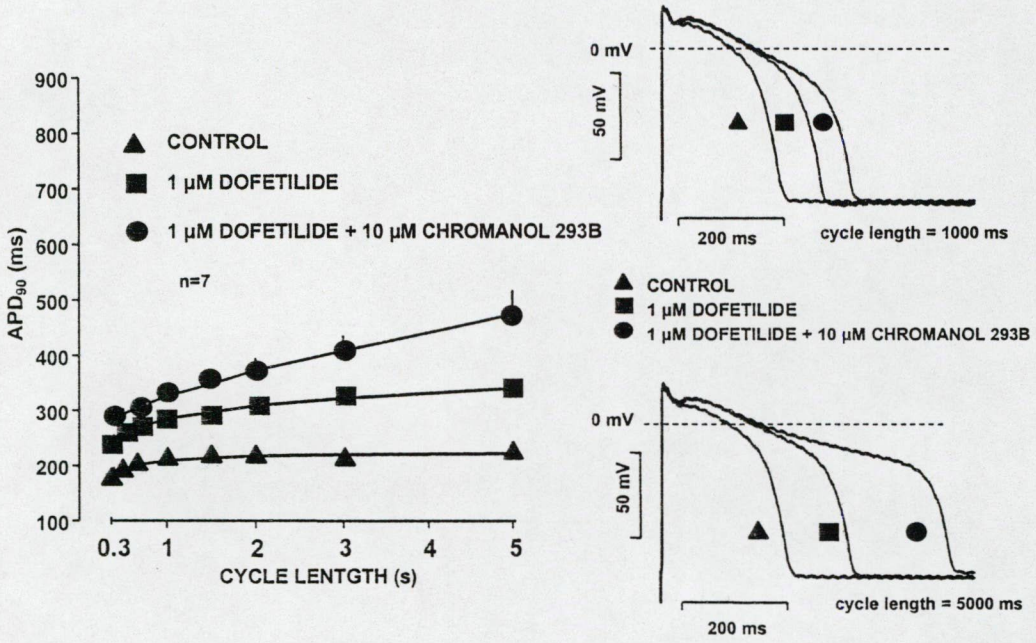


Figure 8: Frequency dependent effect of I_{Kr} (dofetilide) and combined I_{Kr} (dofetilide) and I_{Ks} (chromanol 293B) block on the action potential duration in dog right ventricular papillary muscle. Note that the error bars are often smaller than the corresponding symbols, and the drug combination augmented the reverse rate dependent APD prolongation.

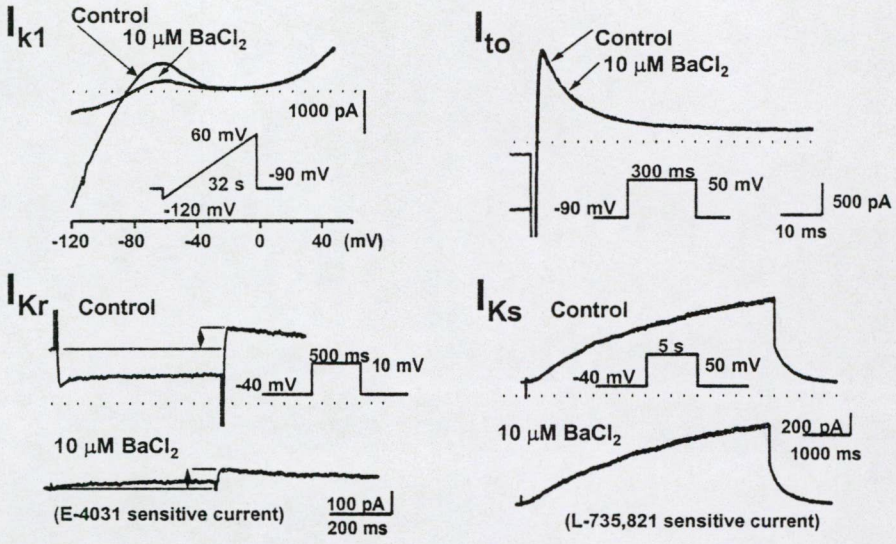


Figure 9: Effect of $BaCl_2$ on the inward rectifier (I_{K1}), transient outward (I_{to}), rapid delayed rectifier (I_{Kr}) and slow delayed rectifier (I_{Ks}) potassium currents in dog ventricular myocytes. For measuring the effect of $BaCl_2$ on I_{Kr} the following protocol was used: (1) current recording under control conditions; (2) current recording in the presence of 10 μM $BaCl_2$; (3) current recording after application of 2 μM E-4031 (in the presence of 10 μM $BaCl_2$). Subtracting (3) from (2) gave the E-4031 sensitive current (i.e. I_{Kr}) in the presence of 10 μM $BaCl_2$. For measuring the effect of $BaCl_2$ on I_{Ks} a similar protocol was used but 0.1 μM L-731,821 was applied for determining I_{Ks}. The applied voltage protocols are shown in the insets, and explained in the text in more detail. In all experiments I_{Ca} was fully blocked by addition of 1 μM nisoldipine. The dotted lines represent the zero current levels.

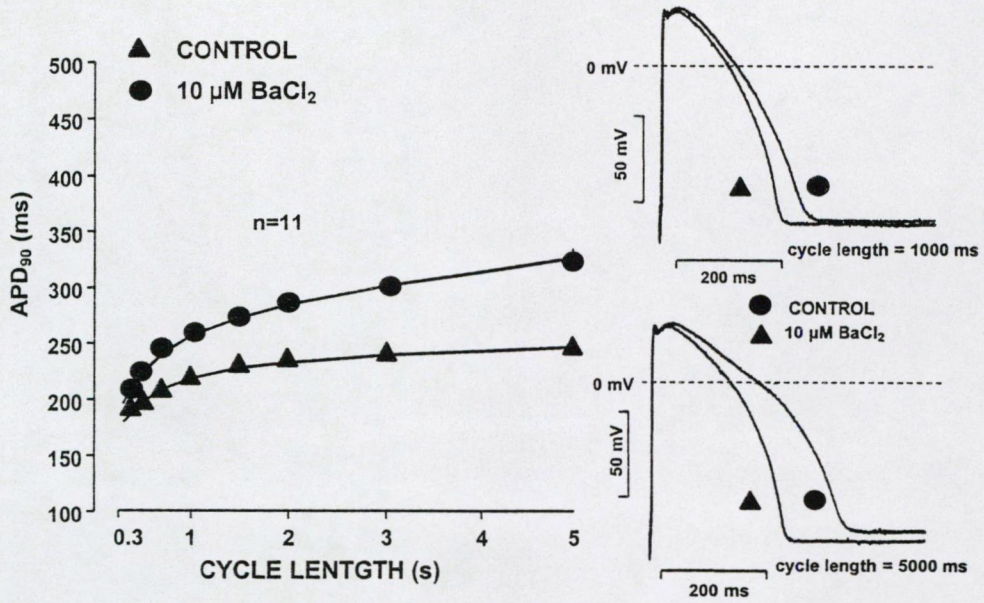


Figure 10: Frequency dependent effect of I_{K1} block ($BaCl_2$) on the action potential duration in dog right ventricular papillary muscle. Note that the error bars are often smaller than the corresponding symbols.

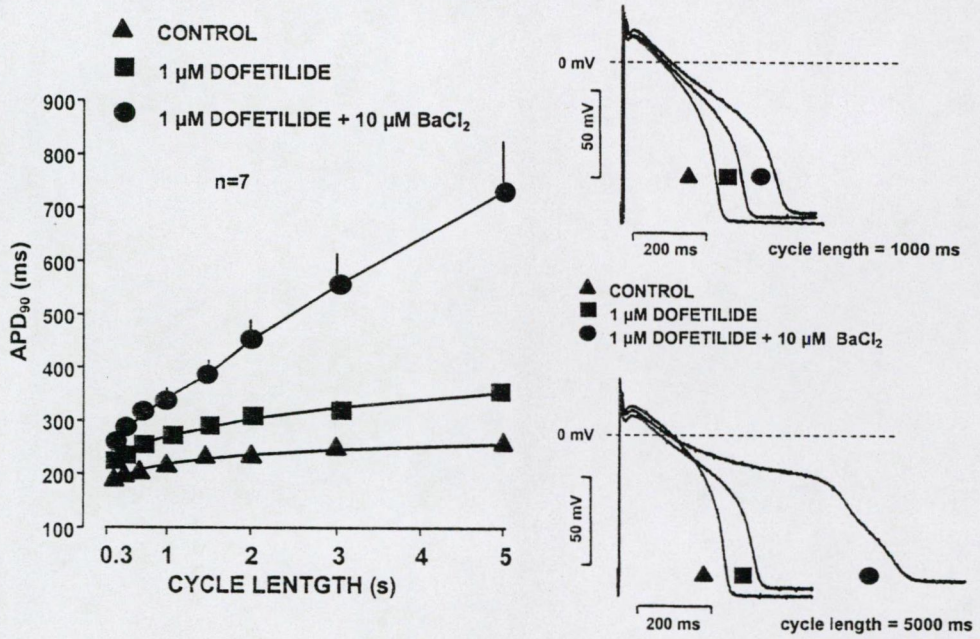


Figure 11: Frequency dependent effect of I_{Kr} (dofetilide) and combined I_{Kr} (dofetilide) and I_{K1} ($BaCl_2$) block on the action potential duration in dog right ventricular papillary muscle. Note that the error bars are often smaller than the corresponding symbols, and the drug combination augmented the reverse rate dependent APD prolongation.

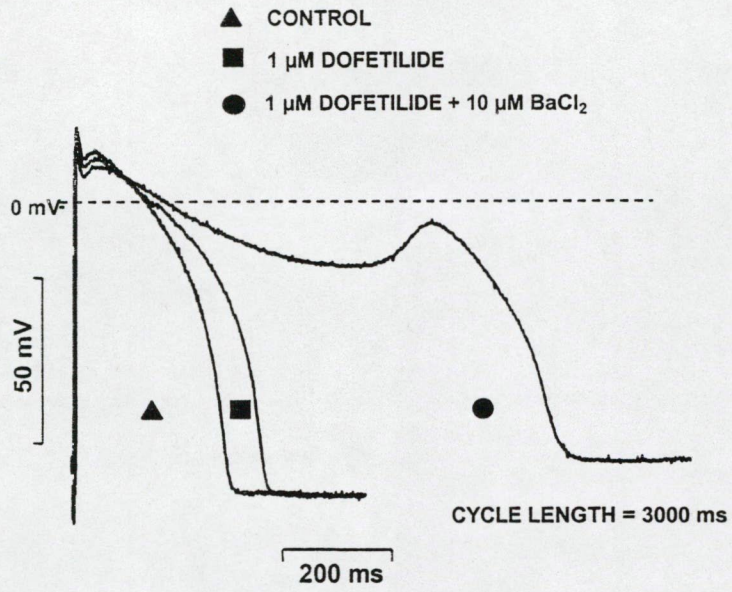


Figure 12: Early afterdepolarization (EAD) evoked by the combined I_{Kr} (dofetilide) and I_{K1} ($BaCl_2$) block in dog right ventricular papillary muscle.

5.3 Goal III: To elucidate the role of I_{Ks} and I_{Kr} in normal human ventricular muscle.

Effects of I_{Ks} and I_{Kr} blockade on human ventricular muscle action potential duration

Chromanol 293B (10 μM), L-735,821 (100 nM) and HMR-1556 (100 nM and 1 μM) reported to selectively block I_{Ks} in cardiac ventricular muscle preparations [18,29,30,31,48,43] in other species produced less than a 9 ms (2.8 %) change in human ventricular papillary muscle action potential duration after 40 min exposure, during continuous pacing at a cycle length of 1000 ms (Figure 13, upper and middle panels). In contrast, d-sotalol (30 μM) and E-4031 (1 μM) expected to selectively block I_{Kr} [18] markedly and significantly increased human ventricular muscle APD under identical conditions (Figure 13, bottom panels). 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 14). Over this range of pacing cycle lengths, either chromanol 293 B (Figure 14, open triangles), L-735,821 (open diamonds) or HMR (100 nM open circles and 1 μM open rectangles) produced no greater than a 12 ms (3.2

%) change in APD, while d-sotalol (closed circles) and E-4031 (closed rectangles) each markedly lengthened human ventricular APD in a reverse frequency-dependent manner.

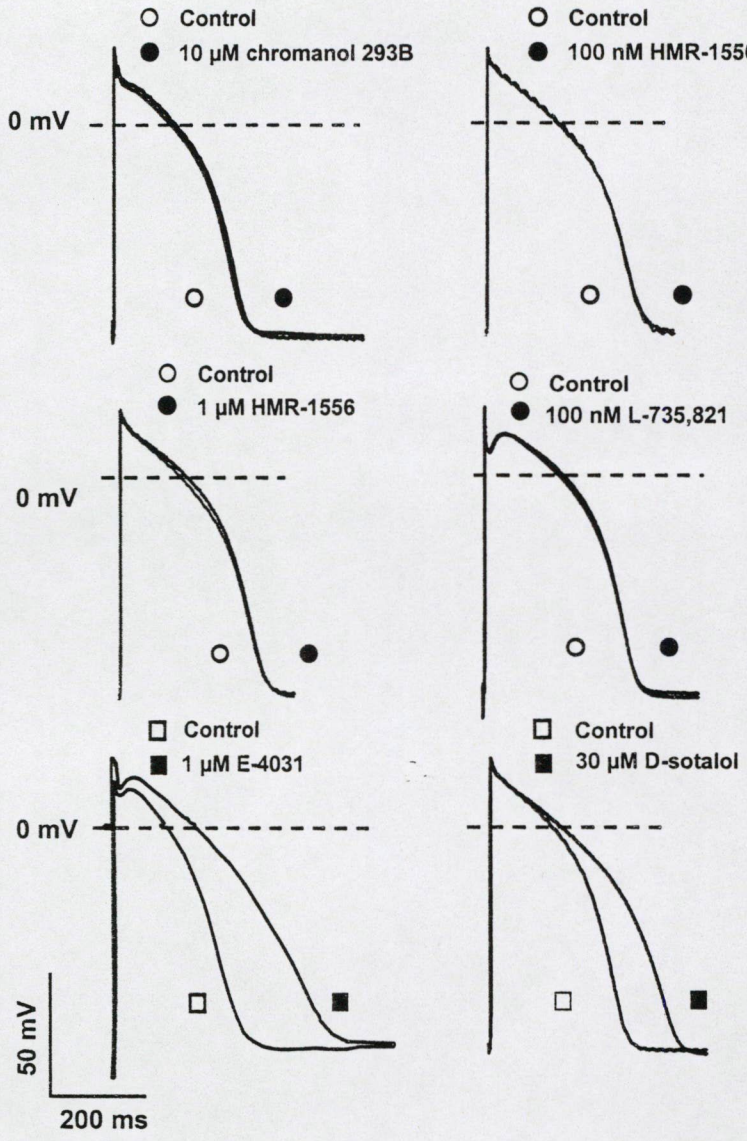


Figure 13: Effects of chromanol 293B, HMR 1556, L-735, 821, E-4031 and d-sotalol on human ventricular muscle action potential duration.

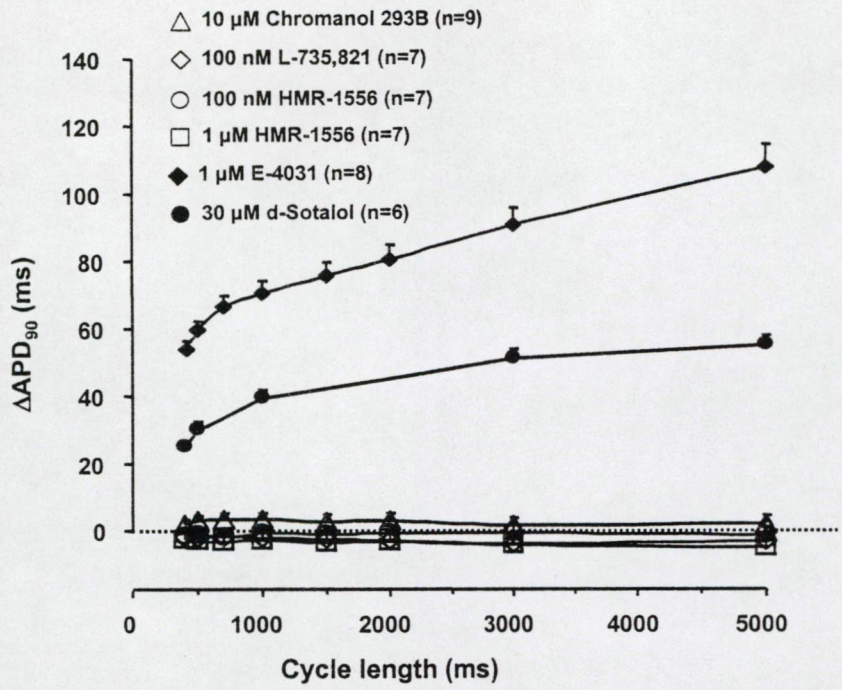


Figure 14: Frequency dependent effects of chromanol 293B, HMR 1556, L-735, 821, E-4031 and d-sotalol on human ventricular muscle action potential duration.

Goal IV. To study the effect of ORM-10103, 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

5.4 Effect of ORM-10103 on the formation of early and delayed afterdepolarisation

Utilizing the conventional microelectrode technique, the effect of ORM-10103 on early (EAD) and delayed (DAD) afterdepolarizations was studied in dog right ventricular papillary muscles and in dog cardiac Purkinje fibers, respectively.

EAD was evoked in the papillary muscle preparation, stimulated at slow cycle lengths (1500-3000 ms), by combination of 1 μM dofetilide and 10 μM $BaCl_2$. As Figure 15 shows 1 and 10 μM ORM-10103 decreased the amplitude of EAD. This effect was reversible upon washout of the compound from the tissue bath containing dofetilide and $BaCl_2$. Similar effect was seen in 5 additional experiments. 3 μM ORM-10103 decreased the amplitude of EADs from 19.3±2.2 to 11.7±2.4 mV (n=6, $p<0.05$). At 10 μM the compound had a more pronounced effect decreasing EADs from 19.4±3.3 to 9.5±4.0 mV (n=4, $p<0.05$).

DAD was evoked in Purkinje fiber preparations superfused with 0.2 μM strophantin for 40 min. In these experiments a train of 40 stimuli was applied with a cycle length of 400 ms in the train. The train was then followed with a stimulation free period of 20 s to observe DAD formation. Following addition of ORM-10103 the amplitude of DADs was decreased (Figure 16). This effect was concentration-dependent; ORM-10103 decreased DAD amplitude at 3 μM concentration from 5.5±0.6 to 2.4±0.8 mV (n=6, $p<0.05$) and at 10 μM from 8.1±2.3 to 2.5±0.3 mV (n=5, $p<0.05$). In two experiments strophantin evoked a run of extra beats appearing after the termination of the stimulus train which was abolished by application of 10 μM ORM-10103.

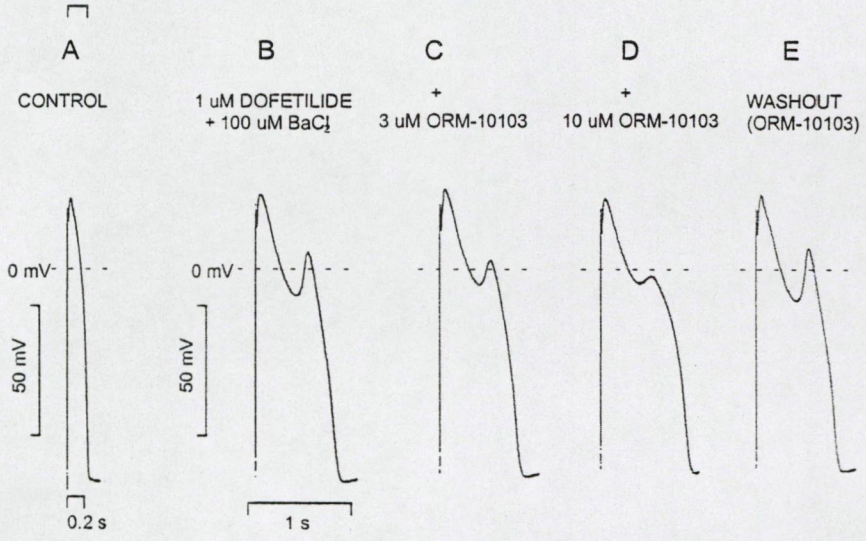
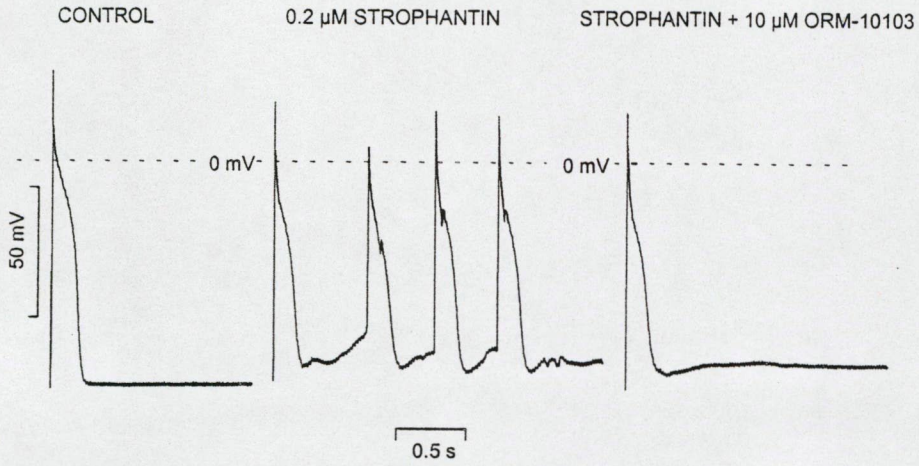


Figure 15: Effect of ORM-10103 on formation of early afterdepolarizations evoked by a combination of 1 μM dofetilide and 100 μM $BaCl_2$



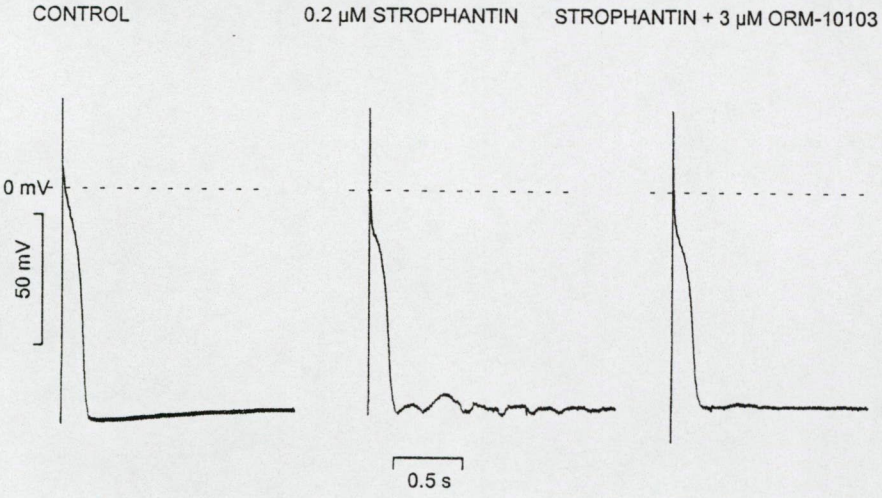


Figure 16: Effect of ORM-10103 on formation of delayed afterdepolarizations evoked by 0.2 µM strophantin.

6. 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), and, 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 interference of their intracellular environment. Changes in parameters of the action potential while studying the effects of different drugs affect changes occuring 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 measured best using the patch clamp technique. However, when measuring the sum of these currents, i.e. the action potential, this method has the above mentioned shortcommings. 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.

6.1 Electrophysiological effects of terikalant

Considering the advantages and disadvantages of the different electrophysiological techniques, we have chosen the measurements with intracellular microelectrodes to investigate the cellular electrophysiological action of terikalant in canine ventricular papillary muscle and Purkinje fiber preparations. Although the therapeutic free plasma level of terikalant is not fully established yet, it lies in the micromolar range [44]. Therefore, the concentrations applied in our experiments reflect therapeutically meaningful concentrations. Our findings showed that the main effect of terikalant is to lengthen APD_{90} (a Class III antiarrhythmic action) in low concentrations which is combined with V_{max} block (Class I +

Class III action) in higher concentrations. RP 58866 and its active enantiomer, terikalant were first considered as selective blockers of the inward rectifier potassium current (I_{K1}) [13]. However, the selectivity of terikalant on I_{K1} was later questioned [45], especially at high concentration. Accordingly, it was found that terikalant also inhibited the rapidly activating delayed-rectifier potassium current (I_{Kr}). Later Williams et al. [46], consistent with the earlier results of Escande et al. [13], but in contrast to the results of Jurkiewicz et al. [44], found that only I_{K1} was significantly affected by 10 μM terikalant, further increasing the uncertainity of the cellular mechanism of terikalant.

Terikalant, like other Class III antiarrhythmic drugs elicits reverse rate-dependent effect on the repolarization both in papillary muscle and Purkinje fibers. Also, as a new finding in our experiments, terikalant in addition to prolonging APD, depressed the maximal upstroke velocity of the action potential (V_{max}) in a frequency-dependent manner. The offset kinetic time constant of V_{max} block produced by the drug was calculated about 3 s, which value appears to be typical for Class I/A antiarrhythmic drugs. The onset kinetics of V_{max} block induced by terikalant in this study was found to be rapid, resembling that of Class I/B antiarrhythmic drugs. Since the present study is the first to demonstrate the effect of terikalant on V_{max} and on its offset and onset kinetics, our results may serve as a basis for its classification. It can thus be concluded that terikalant is a drug with combined Class III and IA antiarrhythmic actions with fairly rapid onset kinetics. The action potential lengthening effect of terikalant can be best explained by its well documented depressing effect on the cardiac transmembrane potassium currents [44]. The use-dependent block of V_{max} is a new finding and reflects a diminution of the inward sodium current in accordance with the modulated receptor hypothesis [47].

In addition to the depression of the maximal upstroke velocity of the action potential (V_{max}) which is due to the sodium channel inhibition, a possible proarrhythmic effect could also appear via increasing the dispersion of repolarization. In fact, since the Cardiac Arrhythmia Supression Trial (CAST) [1], the development of potential antiarrhythmic drugs has been cancelled in cases of those novel compounds that turned out to have sodium channel blocking properties, especially the I/C subgroup. According to our results, besides its well established antiarrhythmic effects, terikalant may increase the proarrhythmic risk as well.

As a conclusion, our study reveals that the investigational drug terikalant, in addition to its potassium channel inhibiting effect, also blocks the fast sodium channels in a rate-dependent manner and it possesses combined electrophysiological mode of action which most likely contributes to its established antiarrhythmic efficacy.

6.2 *The role and interaction of the different potassium currents in the repolarization in dog ventricular muscle*

The most important finding of this study is that I_{Ks} or I_{K1} inhibition resulted in an augmented reverse rate dependent repolarization lengthening in the presence of I_{Kr} block.

In our experiments 10 μM chromanol 293B and 10 μM $BaCl_2$ were applied in order to partially but selectively inhibit I_{Ks} and I_{K1}, respectively. To apply these two agents at higher concentrations in which they would cause complete block of I_{Ks} and I_{K1} was hampered by their known capability to inhibit various other potassium channels [48,49,50]. However, at the concentration of 10 μM, as our experiments demonstrate, they can be regarded as fairly selective blockers of I_{Ks} or I_{K1}, respectively. In spite of this, it can not be entirely ruled out that chromanol 293B and $BaCl_2$ at the applied concentration, can affect other, so far uninvestigated, transmembrane cardiac ion channels or electrogenic transport mechanisms. Further studies would be worthwhile to rule out or establish such possibilities.

These results are in good agreement with the results of previous studies. The recent findings indicate that chromanol 293B alone only slightly lengthens APD in dog papillary muscle, but when APD is lengthened by previous superfusion of E-4031 and veratrine chromanol 293B resulted in a marked prolongation of APD [18], which can be explained by the kinetic properties of I_{Ks}. Accordingly, at longer APD I_{Ks} has more time to be activated and thereby it can more importantly contribute to repolarization. This led us to the conclusion that I_{Ks} could provide an important means of limiting excessive APD lengthening when action potentials are prolonged beyond normal by other mechanisms. In the present work we extended this previous observation, suggesting now that any kind of additive potassium channel block would greatly limit the capability of the ventricular muscle to repolarize.

Vos et al. [51] developed an experimental torsade de pointes arrhythmia model in dogs, in which they found that after complete chronic atrioventricular block downregulation of I_{Ks} and I_{Kr} can be observed [35]. These animals showed longer QTc interval and monophasic APD, and had augmented susceptibility to develop torsade de pointes ventricular tachycardia after administration of certain Class III antiarrhythmic drugs [52]. It is also known that various potassium channels are down-regulated during heart failure [53], resulting in longer APD [54] and in increased risk of proarrhythmia.

In some forms of inherited long QT syndrome, like LQT1 and LQT2 [16,55], loss of functional potassium channel proteins or possible decrease of their density does not always result in marked or manifest QTc lengthening [56,57]. These patients, however, are susceptible to drugs, which can further depress the function of different types of potassium channels, and in such patients this can cause excessive and unexpected APD lengthening, often inducing torsade de pointes tachycardia. These observations strongly argue for the role of the so-called 'repolarization reserve' i.e. that different potassium channels may compensate each other to secure the repolarization process [58].

Similar to our results, very recently, Burashnikov & Antzelevitch [59] reported that attenuation of the repolarization reserve by combining I_{Kr} and I_{Ks} block can result in excessive APD lengthening and development of EADs.

The present experiments may have some important therapeutical and practical implications. Based on these results it seems obvious that in situations where the 'repolarization reserve' is decreased, minor loss of the function of the potassium channels may lead to excessive repolarization lengthening and under certain conditions to development of torsade de pointes arrhythmia. Therefore, patients admitted to hospital should be carefully tested how they respond to Class III antiarrhythmic drugs, and only those patients should receive long-term out of hospital treatment who respond to these drugs with QTc lengthening within the expected range. Similar conclusion can be drawn also from DIAMOND trial [60] as well, since dofetilide treatment proved to be neutral concerning overall mortality, but in a subgroup when mortality was related to QTc lengthening it was found that in patients in whom QTc lengthening was less, the dofetilide induced mortality figures tended to be favourable, but in patients where the QTc lengthening was above average, after dofetilide treatment, the mortality ratio was somewhat increased.

Another possible implication of these results is that we should re-evaluate our safety pharmacology concepts related to drug induced QTc lengthening. Most of the routine safety pharmacology investigations are carried out in channel expression systems or in *in vitro* and *in vivo* tests performed in normal preparations where the 'repolarization reserve' is intact, frequently even at fast heart rates. In these situations the possible proarrhythmic danger of both cardiac and noncardiac investigational compounds is often underestimated, since the drugs may not cause significant APD lengthening under normal conditions, but in patients where the 'repolarization reserve' is impaired, the risk for excessive APD prolongation and consequently for torsade de pointes arrhythmia is greatly enhanced. Therefore, the preclinical

safety pharmacology screenings should be extended to preparations in which 'repolarization reserve' is attenuated.

Goal III. To elucidate the role of I_{Kr} and I_{Ks} in normal 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.

Contrasted to these findings, we demonstrated that E-4031 (1 μM), which blocks I_{Kr} specifically, 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.

Stengl *et al.* [61] 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. They reported that both in dog ventricular myocytes and papillary muscle preparations HMR-1556 (an highly selective I_{Ks} blocker) failed to lengthen APD without prior sympathetic simulation 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 heart failure. Others [62] have suggested that the sensitivity of action potential shortening by I_{Ks} block is enhanced when phosphorylation is increased as expected during elevated sympathetic nervous activity. Clearly, cAMP increases I_{Ks} and may alter its activation and deactivation kinetics so that I_{Ks} may 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 its importance 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. Thus, I_{Ks} normally 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 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 as such I_{Ks} provides a "repolarization reserve" when other outward repolarizing currents are reduced; *e.g.*, due to remodelling of ion currents during heart failure progression [63,64]. This role for I_{Ks} is supported by the finding that specific I_{Ks} block markedly lengthens dog papillary muscle APD after "repolarization reserve" is attenuated by complete I_{Kr} block with 1 μM E-4031 (11,28). Clinical trials would be required to address such speculation.

As an alternative explanation based on a recent study suggests that mutation of KvLQT1 gene may decrease the trafficking of the HERG channel [65]. In this study it was found that coexpression of KvLQT1 gene with the HERG gene increased the membrane immunolocalization of HERG channels by approximately 2-fold emphasizing the complexity of the possible phenotypic consequence of LQT syndroms.

The findings report in this study suggest that development of ventricular antiarrhythmic strategies targeting selective I_{Ks} block are probably not warranted as they would not be expected to effect APD and refractoriness at normal heart rates while reducing "repolarization reserve." If, on the other hand, I_{Ks} could be therapeutically increased (either pharmacologically or genetically), arrhythmia risk might be reduced; 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} expression are downregulated [63,64].

6.3 The electrophysiological effect of the NCX current inhibition

The main finding of this study was that ORM-10103 effectively inhibited the NCX current without affecting I_{Ca}, and this effect was associated with a decrease of the amplitude of EAD and DAD evoked in dog ventricular papillary muscle and cardiac Purkinje fibers, respectively. In our experiments ORM-10103 inhibited the NCX current in dog ventricular myocytes at relatively low concentrations with an estimated IC_{50} of 0.8-0.9 μM. In the same cells ORM-10103, even at high concentration (10 μM), did not influence I_{Ca} measured by the patch clamp and I_{Na} 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 ORM-10103 on EAD and DAD amplitude. Our unpublished data (not shown) indicate that ORM-10103 does not change the inward rectifier potassium current (I_{K1}) and either does not change or slightly decreases the rapid delayed rectifier (I_{Kr}) and transient outward (I_{to}) potassium currents. Therefore, participation of the major potassium

currents is also unlikely in the mechanism whereby ORM-10103 decreases EAD and DAD, 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 a new compound, SEA 0400 [40], does not include the examination of the effect of this compound on arrhythmogenesis, EAD and DAD formation or on action potentials and contractility. KB-R7943 was reported to decrease NCX [66] and abolished experimental arrhythmias [37,38] but this compound can not be considered as specific inhibitor of NCX since it depresses the L-type calcium current, and it has other effects [40]. 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 EAD and DAD may be antiarrhythmic both in the ventricles and the atria [67] 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 [68,69] and the NCX current upregulated [70]. Also, it was considered that on 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 [71]. 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 EAD or DAD.

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 [72,73] can not properly compensate, the resulting excessive Ca^{2+} overload may affect the mitochondria and eventually may cause irreversible myocardial damage.

7. Summary

The most important findings in this PhD thesis are the following:

1. The *in vitro* electrophysiological effects of terikalant, this new investigational antiarrhythmic drug with largely unknown cellular mode of action, were studied which revealed that the investigational drug terikalant, in addition to its Class III potassium channel inhibiting effect, also blocked the fast sodium channels in a rate-dependent manner. In addition, like amiodarone, it possessed combined electrophysiological mode of action which most likely contributes to its established antiarrhythmic efficacy.

2. The possible role and the interaction of different potassium channels in cardiac repolarization in dog ventricular muscle were also investigated. In the dog ventricular muscle when only one type of potassium channel is inhibited, excessive APD lengthening is not likely to occur. This is probably due to the capability of the various potassium channels to substitute each other. Dog ventricular myocytes seem to repolarize with a strong safety margin ('repolarization reserve'). When the normal 'repolarization reserve' is attenuated (due to drugs, remodelling or genetic disorders), the otherwise minimal or moderate potassium current inhibition can result in excessive and potentially proarrhythmic prolongation of the ventricular action potential duration. Also, application of drugs which are able to block more than one type of potassium channel is probably more hazardous than the use of specific inhibitors of a given potassium channel. We should reevaluate our safety pharmacology concept related to possible QT lengthening effect of drugs and apply tests in preparations where the 'repolarization reserve' is impaired instead of using preparations where this reserve is normal.

3. The role of I_{Kr} amd I_{Ks} were examined in human ventricular muscle. As we found earlier, I_{Ks} plays no obvious role in altering action potential repolarization and QT duration at normal heart rates in dog ventricular myocytes. In the present study, this finding is confirmed in isolated human ventricular preparations obtained from the hearts of individuals without heart disease. These findings should not be misconstrued as if I_{Ks} would not play an important role in the normal heart. In the healthy heart it prevents excessive action potential prolongation either following a single long diastolic interval occuring after the compensatory pause that follows a premature ventricular depolarization or during bradycardia.

4. The effect of ORM-10103, 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 ORM-10103 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.

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