The importance of repolarization reserve in mammalian ventricle

PhD Thesis

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Abstracts

I. Kristóf A, Virág L, Kovács PP, Lengyel Cs, Horváth Z, Papp JGy, Varró A
A tranziens kifelé haladó kálium áram szerepe kutya kamrai szívizomban
Cardiologia Hungarica 37:(Suppl.A) p. A60. (2007)

II. Kristóf A, Koncz I, Szél T, Jost N, Biliczki P, Papp JGy, Varró A, Virág L
Diclofenac hatása a repolarizációra kutya kamrai szívizomban. (Effects of diclofenac on the repolarization in dog ventricular muscle)
Cardiologia Hungarica 40:(Suppl.G) p. G44. (2010)

III. Kristóf A, Kohajda Zs, Szél T, Husti Z, Baczkó I, Varró A, Jost N, Virág L
Transient outward potassium current in dog atrial preparations. (A tranziens kifelé haladó káliumáram kutya pitvari preparátumokon)
Cardiologia Hungarica 41:(Suppl.F) p. F35. (2011)

ABBREVIATIONS

APD action potential duration

EAD early afterdepolarizations

ECG electrocardiography

GTP guanosine triphosphate

I_{CaL} L-type calcium current

I_{Kr} rapid component of the delayed rectifier potassium current

I_{Ks} slow component of the delayed rectifier potassium current

I_{K1} inward rectifier potassium current

I_{to} calcium independent transient outward current

K_2ATP adenosine 5′-triphosphate dipotassium salt

LQTs long QT syndrome

NSAID non-steroid anti-inflammatory

SEM standard error mean

SWORD Survival With Oral d-Sotalol

TdP torsade de pointes

CONTENTS

1. INTRODUCTION

1.1 Repolarization reserve

It has been well known since the SWORD (Survival With Oral d-Sotalol) study [1] that antiarrhythmic drugs may induce a polymorphic ventricular tachycardia called torsade de pointes (TdP), which may degenerate into fatal ventricular fibrillation. This proarrhythmic effect related to the QT_c prolonging property of these drugs, which are mostly class III antiarrhythmics. However, not only antiarrhythmics but also a wide variety of noncardiac agents, including antibiotics, antipsychotics antidepressants and antihistamines, possess proarrhythmic QT_c prolonging property. Albeit the incidence of TdP or sudden cardiac death induced by non-cardiac drugs is low, several of them, such as cisapride and terfenadine were withdrawn from the market. Therefore, in order to develop new drugs with less proarrhythmic potency it is important to understand the mechanism of the repolarization abnormalities leading to this life-threatening arrhytmias.

Drug-induced TdP arrhythmia is a complex phenomenon. It is not related purely to the QT_c prolongation efficacy of the particular drug. In fact, QT_c prolongation is a poor marker of the proarrhythmic risk. Often, drugs that just moderately prolong QT_c interval were found to be more torsadogenic than some agents which substantially lengthen QT_c [2,3,4]. The risk factors of these arrhythmias include high drug concentration as well as dispersion of QT intervals, female gender, hypokalemia, hypomagnesemia, bradycardia, congenital long QT syndrome and also diseases such as cardiac hypertrophy or congestive heart failure [5,6,7,8,9,10].

According to the theory of "*repolarization reserve"* there are multiple different potassium currents, which implement a redundant mechanism to accomplish repolarization process in the normal heart. Therefore, in a healthy heart pharmacological block of a potassium current does not result in dangerous QT_c prolongation. Thus, this mechanism provides a strong safety reserve for repolarization. However, in pathological conditions where the density of one or more type of potassium current is attenuated by congenital channelopathies, remodeling or by any other heart disease, inhibition or impairment of another potassium current may cause excessive lengthening of the action potential duration (APD) and the QT_c interval leading to harmful cardiac arrhythmias such as TdP [11,12,13].

1.2 Main K^+ currents influencing repolarization process and the repolarization reserve

Ventricular repolarization in the heart is controlled by a fine balance of several inward and outward transmembrane ion currents, electrogenic exchanger and pump mechanisms. Since these currents are in continuous and dynamic interaction with each other, the repolarization mechanism is very complex. Many of these ion currents depend not only on the membrane potential but also on the intracellular Ca^{2+} level, which is in continuous change with the Ca^{2+} release during the action potential course. Therefore, it is very difficult to investigate the role of an individual current in the repolarization process.

The rapid component of the delayed rectifier potassium current (I_{Kr}) is one of the major ion currents responsible for the repolarization of the action potential in ventricular muscle [14]. The kinetic properties of this current are similar in rabbit, dog and human[15,16,17,18]: I_{Kr} activates relatively rapidly at positive membrane potentials and deactivates slowly during repolarization at negative voltages. The behavior of this gate however, is very unusual; the inactivation of this current is faster than the activation process. Therefore, upon depolarization the great majority of the channels are inactivated before the activation process taking place. This unusual property of the inactivation gate is the main reason of the inward rectification of this current [19], which substantially limits the amplitude of I_{Kr} at positive membrane potentials – during the plateau of the action potential. The activated small I_{Kr} current, however, can influence the slope of the plateau phase. As the membrane potential repolarizes to below 0 mV, the channels recover from inactivation leading to reactivation of a considerable amount of I_{Kr} current that further increases the rate of repolarization. The deactivation of I_{Kr} is slow enough at the diastolic potential to enable accumulation of the current at faster heart rate or in case of a premature action potential. Therefore, the contribution of I_{Kr} current to action potential repolarization is rate-dependent.

The slow component of the delayed rectifier potassium current (I_{Ks}) activates much more slowly than I_{Kr} current but it deactivates relatively rapidly in dog ventricle [20]. In rabbit it was reported that the density of this current is higher but the activation and deactivation kinetics of the current is similar to that found in dog [15,16]. In human ventricle the kinetic properties of I_{Ks} current is similar to that measured in dog and rabbit but the density of the current is lower in human than in dog or in rabbit ventricular myocytes [21]. Since I_{Ks} activates slowly (with a time constant of 900-1000 ms) and the duration of plateau phase is not longer than 150-200 ms and also the plateau potential is usually not more than 20 mV, only very little current is expected to flow through these channels during the plateau

phase of a normal action potential. Therefore, I_{Ks} play a minor role in the repolarization of the normal ventricular muscle but more I_{Ks} current is expected to activate when action potential is abnormally prolonged in pathophysiological conditions, due to the longer plateau phase, which in turn limits the excessive repolarization lengthening. This negative feed-back mechanism provides an important safety factor for the repolarization process and thus, I_{Ks} has an important role in cardiac ventricular repolarization reserve [22,23].

The inward rectifier potassium current (I_{K1}) flows through several different ion channels such as Kir2.1, Kir2.2, Kir2.3 and Kir2.4. The density of I_{K1} is species dependent: it is relatively weak in human compared to that found in dog [18]. Because of the inward rectification property of this current the conductivity of these channels is much higher around and below the resting potential than at more positive potential ranges where the direction of the current is outward, which is due to voltage dependent channel blockade by intracellular Mg^{2+} and polyamines [24, 25]. Thus, during diastole the K^+ permeability is very high, which keeps the resting potential at adequately negative level – close to the K^+ equilibrium potential, and due to the strong blockade of the current at more positive to -20 mV the current level at this potential regions is close to zero, which helps to maintain the potential of the plateau phase. However, the current is considerably higher at more negative to -20 mV, therefore it carries repolarizing current at the later phase of repolarization and it is an important contributor to the repolarization reserve.

The calcium independent transient outward current (I_{to}) is activated by depolarization to more positive than -20 mV and inactivates rather rapidly during the plateau potential. The kinetics of recovery from inactivation is either rapid (human, dog) or slow (rabbit) depending on the pore forming subunit. Various beta subunits such as KChIP2 or DPP also modify the magnitude and the kinetic properties of the current [26,2718]. I_{to} is present in dog, human and rabbit but absent in guinea-pig and pig [28,29,30,31,3218]. In rabbit most probably Kv1.4 tetramers form the pore forming subunit of the channel. Therefore, due to the very slow recovery kinetics the current is largely inactivated at the normal heart rate of rabbit. In dog and human I_{to} is mediated mostly by alpha subunits constructed by Kv4.3 tetramers, which possess rapid recovery kinetics. Thus, majority of the channels can be active at normal and even at fast heart rates.

Studying the contribution of I_{to} to cardiac repolarization and to the repolarization reserve is very problematic due to the lack of selective blocker of the current. Earlier both experimental and mathematical modeling works studying the role of I_{to} in repolarization led to conflicting results; 4-aminopyridine in low concentration (1 mM/L) shortened [33,34], while

higher dose (3-5 mM/L) lengthened [35,36] the action potential duration in ventricular myocytes. The interpretation of these effects is difficult, because on the one hand 4-aminopyridine is not a specific blocker of I_{to}, it has been reported to alter several other important transmembrane ion currents [37]. On the other hand, I_{to} blockade may modulate the potential of the plateau phase, which in turn may affect other important transmembrane ion currents such as L type calcium current and the rapid delayed rectifier potassium current, influencing indirectly the repolarization of the action potential. Therefore, blockade of I_{to} may lead to either lengthening or shortening of the action potential. It has also been reported that in guinea pig heart, the introduction of I_{to} by adenovirus vectors produces a reduction of action potential duration [38].

It has been shown in a modeling work that the I_{to} conductance does not significantly affect the action potential duration in the Luo-Rudy model [39]. On the other hand, another research group reported that I_{to} tended to lengthen APD if the current density was low, while it shortened APD when the I_{to} density was high [40].

Now, it is generally accepted that I_{to} plays important role only in the early repolarization phase of the action potential because the current inactivates relatively early during the plateau. It was also suggested, as mentioned earlier, that the current can modulate the plateau potential, and consequently may affects other important transmembrane ion currents influencing indirectly the ventricular repolarization [41,42]. However, the contribution of I_{to} to the repolarization of the action potential and to the repolarization reserve is not well understood and still controversial.

1.3 Pathological changes associated with reduced repolarization reserve

Heart failure

The heart failure is a very common disease in the developed countries with worse life expectancy than cancer. Common causes of heart failure include coronary artery disease, myocardial infarction, high blood pressure, atrial fibrillation, valvular heart disease, and cardiomyopathy. However, about half of these patients die due to sudden cardiac death caused by fatal ventricular arrhythmias. It has been shown that the ventricular action potential is prolonged in heart failure patients and in animal models [43,44,45]. The most consistent findings are the downregulation of transient outward and the slow delayed rectifier potassium currents [44,46,47,48]. The inward rectifier potassium current is also found to be reduced in some studies [44,46,36,49]. The results concerning the rapid delayed rectifier potassium

current are more inconsistent. It has been found that I_{Kr} remained unaltered in heart failure [44,46,50], while others reported that I_{Kr} is downregulated in animal heart failure models [47,50]. The downregulation of these repolarizing ion currents together with upregulation of the slowly inactivating late sodium current found in heart failure [51] imply impairment of repolarization reserve and elucidate the prolonged action potential in heart failure patients, which favors the formation of early afterdepolarizations (EAD) and life-threatening ventricular tachyarrhythmias.

Diabetes mellitus

Diabetes mellitus, both type 1 and type 2, is related to increased risk of sudden cardiac death [52,53], which is not attributed to atherosclerosis, hyperlipidaemia, heart failure or other pathological causes. Prolongation of the QT_c interval and increased QT_c dispersion were reported in type 1 diabetes mellitus patients [54,55], which might be in the background of the increased cardiac mortality in diabetes. Nevertheless, the cellular mechanism of the QT_c interval lengthening and the increased QT_c dispersion in type 1 diabetes mellitus patients is not understood well. Most of the earlier studies investigating the changes of transmembrane ion currents in diabetes were performed in rats [56,57,58,59]. However, the rat ventricular action potential waveform and the repolarization process are different from that found in larger mammals such as rabbit, dog and human; due to the much higher heart rate the action potential is short in duration with lack of plateau phase. Lengyel et al. observed reduction of the density of transient outward and slow delayed rectifier potassium currents in diabetic dogs [60]. Other ion currents such as inward rectifier potassium current, rapid delayed rectifier potassium current and the L-type calcium current did not change in the diabetic animals. In other works reduction of the density of I_{Kr} was reported in diabetic rabbits but the authors did not demonstrate how the other ion currents behave in diabetes [61,62]. Therefore, the decreased density of I_{to}, I_{Ks} and I_{Kr} observed in various animal models may induce the repolarization abnormalities and the mild QT prolongation found in diabetes mellitus patients, which may lead to increased risk of arrhythmias and sudden cardiac death.

Long QT syndrome

Congenital Long QT syndrome (LQTs) may substantially attenuates the repolarization reserve in the heart. A great number of mutations of ion channel genes leading to impairment of the repolarization process increase the risk of episodes of torsade de pointes, which may degenerate into ventricular fibrillation. These mutations are loss of function mutations causing

reduction of repolarizing currents such as I_{Ks}, I_{Kr}, I_{K1} or gain of function mutations such as mutation of alpha subunit of the sodium channel (SCN5A), which slows down the inactivation of sodium current increasing the late sodium current (I_{NaL}). Mutation of KCNQ1 (KvLQT1) and KCNE1 (MinK) genes result in loss of I_{Ks} function in LQT1 and LQT5, while in LQT2 and LQT6 loss of I_{Kr} function occurs due to KCNH2 (ERG) and KCNE2 (MiRP1) gene mutations, respectively. Mutation of KCNJ2 gene ($K_{ir}2.1$) in LQT7 syndrome lead to loss of I_{K1} function. LQT3 syndrome is characterized by gain of function mutations of SCN5A gene ($Na_v1.5$) [63].

The long QT syndrome is not necessarily a hereditary disorder. The most frequent form of acquired LQT syndrome is due to application of medications. It is well known that lot of antiarrhytmic agents and also other non-cardiac drugs (such as some antihistamines, antibiotics, psychotropic drugs, etc.) possess K^+ channel blockade property and exhibit torsadogenic activity.

Not only genetic disorders, medications and diabetes but also such risk factors as hypokalemia, hypomagnesemia, female gender, hypothyroidism, and body temperature abnormalities may attenuate the repolarization reserve. In failing heart – as described above – and also in hypertrophic heart the downregulation of several ion currents may also diminish repolarization reserve. A loss of function mutation of an ion channel often does not prolong ventricular repolarization and the QT interval. However, a subsequent drug application or another risk factor may considerably diminish repolarization reserve, increase QT interval and the risk of potentially fatal arrhythmias [64].

Top athletes

In competitive athletes, the hard training activities result in a reversible cardiac hyperthrophy called athlete's heart. Cardiac hyperthrophy, however, leads to electrical remodeling associated with downregulation of several potassium currents including I_{Ks}, a repolarizing current that has a uniquely important role in cardiac ventricular repolarization reserve that results in decreased repolarization reserve in the heart and may increase the risk of arrhythmias and sudden cardiac death [65]. In fact the incidence of sudden cardiac death in the population of young athletes is 2–4 times more than in the age-matched population not involved in competitive sport [66]. However, a mild decrease of the repolarization reserve alone may not increase considerably the risk of arrhythmia but together with other important factors influencing repolarization reserve and the arrhythmogenesis may occasionally lead to sudden cardiac death. These risk factors may be other cardiac diseases, hypokalemia, doping

and seemingly harmless medications, such as non-steroid anti-inflammatory (NSAID) drugs often used by athletes to alleviate sports injuries related pain. There are reports of increased cardiovascular risks of NSAIDs application [67,68]. Diclofenac is such a drug that is widely used in large doses [69]. One may speculate that direct cardiac electrophysiological effects of this drug on potassium channels may lengthen ventricular repolarization leading to dangerous arrhythmias, which contribute to the higher incidence of sudden cardiac death among young athletes.

2. MAJOR SPECIFIC EXPERIMENTAL GOALS

(1) To investigate the effects of the widely used non-steroid anti-inflammatory drug diclofenac in different dog ventricular preparation:

(a) on the ventricular action potential

(b) on the 4-aminopyridine sensitive transient outward potassium current (I_{to})

(c) on inward rectifier potassium current (I_{k1})

(d) on the rapid and slow components of the delayed rectifier potassium current (I_{Kr} and I_{Ks})

(e) on the L-type calcium current (I_{Ca}).

(2) To investigate and analysis of the contribution of I_{to} to repolarization in dog ventricular myocardium.

(3) To investigate the electrophysiological changes induced by experimental (alloxan-induced) type 1 diabetes in the rabbit.

3. METHODS

3.1. Experimental animals

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 the Committee on Animal Research of the Animal Health and Animal Welfare Directorate, Hungary (15.1/01031/006/2008) as well as by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (approval number: I-74-5-2012) and by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development (authority approval number XIII/1211/2012).

3.1.1. Induction of diabetes mellitus by alloxan

Male New Zealand white rabbits (n = 19), weighing 1.5–2.0 kg, were used in this study. Diabetes mellitus was induced in 10 rabbits by infusion of a single intravenous dose of alloxan (145 mg kg)$^{-1}$ alloxan monohydrate, Sigma, St Louis, MO, USA) into the ear vein under pentobarbital anaesthesia (26–30 mg kg^{-1} i.v., Nembutal; CEVA, Paris, France), after fasting overnight. To reduce the risk of nephrotoxicity, alloxan was reconstituted and injected in 20 ml of 0.9% saline. To counteract initial hypoglycaemia, glucose (4.5–5.0 g kg^{-1} i.v.) was given 60 min after the injection of alloxan, and 5% glucose was provided in the drinking water of the animals for the initial 24 h. Blood glucose was measured twice weekly from blood samples obtained from the ear vein. The untreated animals were used as controls. Body weight and plasma glucose levels were determined in each animal at the beginning as well at the end of the experiment (just prior to the electrophysiological analysis). This period lasted 3 weeks in rabbits. We opted for this 3-week model based on previous studies performed in streptozotocine-induced rat/mouse models, where the electrophysiological remodelling effects were investigated after 2–4 weeks of diabetic period [70,71].

3.2. Electrocardiography

Diabetes mellitus rabbits

Conventional ECG recordings were taken for each rabbit under anaesthesia at the beginning and end of the experiment. All leads were sampled by an ECG signal processing system (Haemosys; Experimetria, Budapest, Hungary) under the control of a personal computer. Following analogue-to-digital conversion, the data were stored digitally and analysed off-line. ECG parameters (RR, PQ, QRS, QT and QTc intervals) were determined manually using cursors. QT intervals were measured on lead II from the QRS onset to the end of the T wave. QTc intervals were derived according to Carlsson's formula [QTc = QT) 0.175(RR) 300)] [72]. ECG parameters were averaged from three consecutive complexes analysed always by a single observer.

3.3. Action potential measurement

New-Zealand white rabbits and adult mongrel dogs of either sex were used. Endocardial preparations (papillary or trabecular muscles) were isolated from the right ventricle of hearts removed from anaesthetized (sodium pentobarbital, 30 mg/kg iv.) animals. Free running false tendons of Purkinje fibres were excised from the right or the left ventricles and subepicardial muscle preparations obtained from the right ventricles of dog 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. Each preparation was initially stimulated [HSE (Hugo Sachs Elektronik) stimulator type 215/II, March-Hugstetten, Germany] at a basic cycle length (BCL) of 1000 ms (500 ms for Purkinje fiber), using 2 ms rectangular constant voltage pulses isolated from ground and delivered across bipolar platinum electrodes in contact with the preparation. Transmembrane potentials were recorded using conventional microelectrode techniques. Microelectrodes filled with 3M KCl and having tip resistances of 10-20 Mohm were connected to the input of a high impedance electrometer (HSE microelectrode amplifier type 309), which was connected to ground. The voltage outputs from all amplifiers were displayed on a dual beam memory oscilloscope (Tektronix 2230 100 MHz digital storage oscilloscope, Beaverton, OR, USA). Data acquisition was performed with an ADA 3300 analogue-to-digital board (Real Time

Devices Inc., State College, PA, USA.) using a maximum sampling rate of 40 kHz. The maximum diastolic potential, action potential amplitude, maximum upstroke velocity (V_{max}) and the action potential duration (APD) measured at 50 and 90% repolarization (APD_{50-90}) were obtained using software developed in our department (HSE-APES) on an IBM 386 microprocessor based personal computer. After control measurements the preparations were superfused for 40 - 60 min with Tyrode's solution containing the compound under study, and then the electrophysiological measurements were resumed.

3.4. Ion current measurements

Isolation of rabbit myocytes

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) HEPES buffered Tyrode's solution. The heart was mounted on a modified, 60 cm high Langendorff column and perfused with oxygenated and pre-warmed (37 °C) HEPES buffered Tyrode's solution. After washing out blood (3-5 min) it was perfused with Ca^{2+}-free solution (5 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 Protease E (SIGMA) with 0.1% Albumin. 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's solution. This procedure was repeated twice. The cells were stored at room temperature in HEPES buffered Tyrode's solution.

Isolation of dog myocytes

Ventricular myocytes were enzymatically dissociated from dog hearts, removed from anaesthetized (sodium pentobarbital, 30 mg/kg iv.) animals. The hearts were immediately placed in cold (4 °C) HEPES buffered Tyrode's 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) HEPES buffered Tyrode's solution (10 min), (2) Ca^{2+}-free solution (10 min), and (3) Ca^{2+}-free solution containing collagenase (0.3 – 0.4 mg/ml, Sigma type I) and 33 μM $CaCl_2$ (15 min). Protease (0.04 mg/ml, Sigma type XIV) 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 in Ca^{2+}-free solution supplemented with $CaCl_2$ (1 mM) for 15 min. Next, these tissue samples were gently agitated in a small beaker to dislodge single myocytes from the extracellular matrix. 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 were maintained at 13-14 °C prior to experimentation.

Compositions of solutions used for cell isolation: i) HEPES buffered Tyrode's 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).

Patch clamp technique

One drop of cell suspension was placed in a transparent recording chamber mounted on the stage of an inverted microscope (IX51, Olympos, Tokyo, Japan), and individual myocytes were allowed to settle and adhere to the chamber bottom for at least 10 minute before superfusion was initiated. Only rod shaped cells with clear cross striations were used. HEPES buffered Tyrode's solution 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 (a typical composition in mM: K-aspartate 100, KCl 40, K_2ATP 5, $MgCl_2$ 5, EGTA 4 $CaCl_2$ 1.5 and HEPES 10 at pH 7.2 by KOH). Cell capacitance was measured by integration of the capacitive transient divided by the amplitude of the voltage step (10 mV). The capacitive transient was evoked by a 10 mV hyperpolarizing pulse from -10 mV, while the holding potential was -90 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 selective I_{Kr} blockers, such as E-4031 (1 μM, Institute for Drug Research, Budapest, Hungary) and dofetilide (100-500 nM, Ercom Ltd, Budapest, Hungary) or by applying selective I_{Ks} blockers: L-735,821 (100 nM, a gift from Merck-Sharpe & Dohme, West-Point, PA, USA), chromanol 293B (30 μM, Aventis Pharma, Frankfurt, Germany) or HMR 1556 (500 nM, Aventis Pharma).

In another set of experiments in whole-cell voltage clamp mode an action potential waveform, which was previously recorded from a dog left ventricular epicardial slice with sharp microelectrode by conventional microelectrode technique, was delivered as command potential with a frequency of 0.2 Hz. After current traces were recorded under control conditions 100 μM chromanol 293B was applied. Chromanol 293B sensitive current was defined as I_{to} by subtracting the post-drug curve from the pre-drug one. I_{Ks}, I_{Kr}, and I_{Ca} currents were pharmacologically blocked throughout the measurements.

The L-type calcium current was recorded in HEPES buffered Tyrode's solution containing 3 mM 4-aminopyridine in order to block the transient outward potassium current (I_{to}) and a special K^+ free pipette solution was used (composition in mM: CsOH 100, CsCl 20, TEACl 20, MgATP 5, HEPES 10, EGTA 4, $CaCl_2$ 1.5, GTP 0.1, the pH was adjusted to 7.2 with aspartic acid).

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. 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, after low-pass filtering at 1 kHz, using a 333 kHz analogue-to-digital converter (Digidata 1200 and 1322A, Axon Instruments) under software control (pClamp 8.0 and 10.0, Axon Instruments Inc.). Analyses were performed using Axon (pClamp 8.0 and 10.0, Axon Instruments Inc.) software. All patch-clamp data were collected at 37 ˚C.

3.5. Statistical analysis

Results were compared using one-way analysis of variance (ANOVA) and/or Student's t-tests for paired or unpaired data as appropriate. Differences were considered significant when $p < 0.05$. Data are expressed as mean ±SEM.

4. RESULTS

4.1. Investigation of the effect of NSAID drug diclofenac in cardiac repolarisation in dog ventricular preparations

4.1.1. Effects of diclofenac on action potential

The effects of diclofenac on action potential configuration were studied in dog right ventricular papillary muscle and Purkinje fibers. Small but statistically significant action potential lengthening was induced by diclofenac (20 µM) at a basic stimulation frequency of 1 Hz (from 222.3±4.1 ms to 232.2±3.4 ms, n=13, $p<0.05$). The maximum upstroke velocity was also decreased by the drug (control: 168.8±15.7 V s^{-1}, 20 µM diclofenac: 136.6±13.2 V s^{-1}, n=13, $p<0.05$). To study the rate-dependent effect of the drug on APD_{90}, the preparations were stimulated at cycle lengths ranging from 300 to 5000 ms. Under these circumstances diclofenac produced a slight rate-independent APD prolongation (Figure1A).

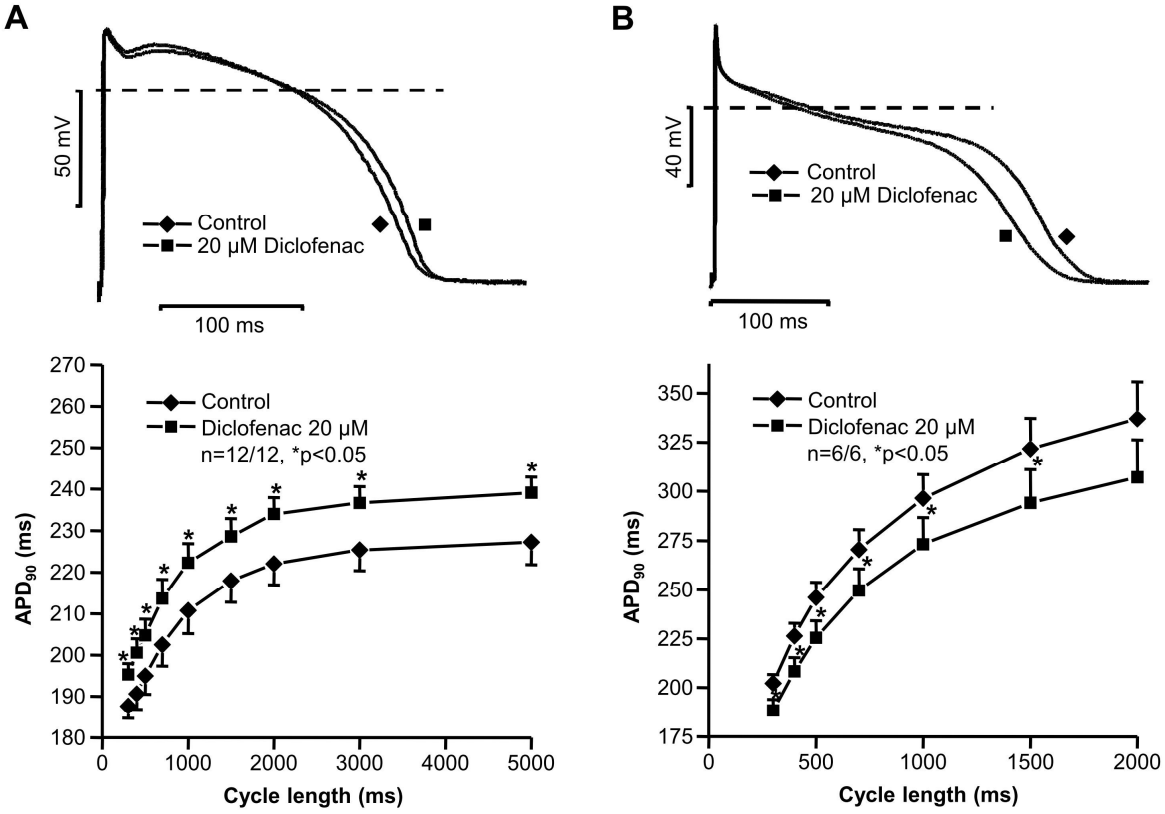

Figure 1. Representative superimposed records (top) and cycle length dependent changes in action potential duration (APD_{90}) (bottom) demonstrating the effect of 20 µM diclofenac on action potential configuration in canine right ventricular muscle preparation (A) and in Purkinje fiber (B). Data are expressed as mean ± SEM, n=number of measurements/number of animals.

In dog Purkinje fibers, however, the drug significantly shortened the action potential duration (from 248.1±10.9 ms to 230.8±9.7 ms, n=6, $p<0.05$) and decreased V_{max} (from 673.9±8.5 V s^{-1}, t o 562.4±27.0 V s^{-1}, n=6, $p<0.05$) at basic cycle length of 500 ms indicating a sodium channel blocking property of the drug. The shortening of APD_{90} was rate-independent (Figure 1B).

The influence of diclofenac on action potential repolarization in preparations with impaired repolarization reserve was also investigated. Repolarization reserve was greatly attenuated by the application of 30 µM $BaCl_2$, which partially blocks I_{K1} in dog right ventricle (Virág *et al*., 2009) [73]. $BaCl_2$ lengthened APD in a reverse rate-dependent manner (Figure 2). In the presence of $BaCl_2$, 20 µM diclofenac induced a marked further lengthening relative to the APD_{90} values measured after the administration of $BaCl_2$ (diclofenac: 309.8±15.2 ms vs. $BaCl_2$: 283.5±15.3 ms, n=11, $p<0.05$, at cycle length of 1000 ms), i.e. APD lengthening effect of diclofenac was significantly augmented in preparations where the "repolarization reserve" was attenuated by previous application of $BaCl_2$ (Figure 2 A). Under these circumstances the drug produced reverse rate-dependent APD prolongation (Figure 2B).

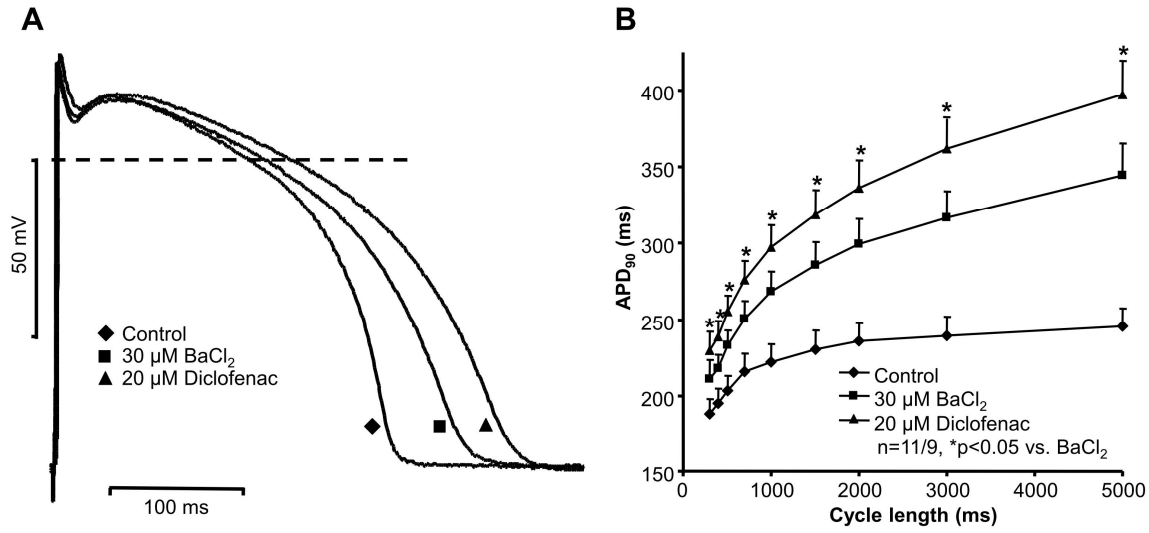

Figure 2. Effect of diclofenac on action potential repolarization in canine right ventricular preparations with impaired repolarization reserve. (A) Representative superimposed action potentials recorded from canine right ventricular muscle preparation at cycle length of 1 s. (B) Cycle length dependent changes in APD_{90} measured under the specified experimental conditions in canine right ventricular muscle preparation. Data are expressed as mean ± SEM, n=number of measurements/number of animals.

4.1.2. Effects of diclofenac on transmembrane ion currents

The effects of the drug on the 4-aminopyridine sensitive transient outward (I_{to}), the inward rectifier (I_{K1}), the rapid and slow delayed rectifier (I_{Kr} and I_{Ks}) potassium currents and on the L-type calcium current (I_{Ca}) were investigated in dog ventricular myocytes. As shown

in Figure 3A and 3B, diclofenac (even at 50 µM concentration) did not influence I_{to} or I_{K1} currents. I_{to} current was activated by 1000 ms long depolarizing voltage pulses from the holding potential of -80 mV to test potentials ranging from -20 to 60 mV with a pulse frequency of 0.2 Hz. I_{K1} current was measured as the steady-state current level at the end of the 300 ms long voltage pulse in the voltage range of -100 to 0 mV with a pulse frequency of 0.33 Hz. The holding potential was -90 mV.

I_{Kr} and I_{Ks} were measured using 1000 ms (I_{Kr}) or 5000 ms-long (I_{Ks}) test pulses between -30 mV and 50 mV (I_{Kr}) or -20 to 50 mV (I_{Ks}). The holding potential was -80 mV and during I_{Kr} measurements 500 ms long prepulse to -40 mV was applied in order to ensure the baseline region. The pulse frequency was 0.05 Hz (I_{Kr}) or 0.1 Hz (I_{Ks}). The decaying tail current at -40 mV after the test pulse was assessed as I_{Kr} or I_{Ks}. The top panels of Figure 4A

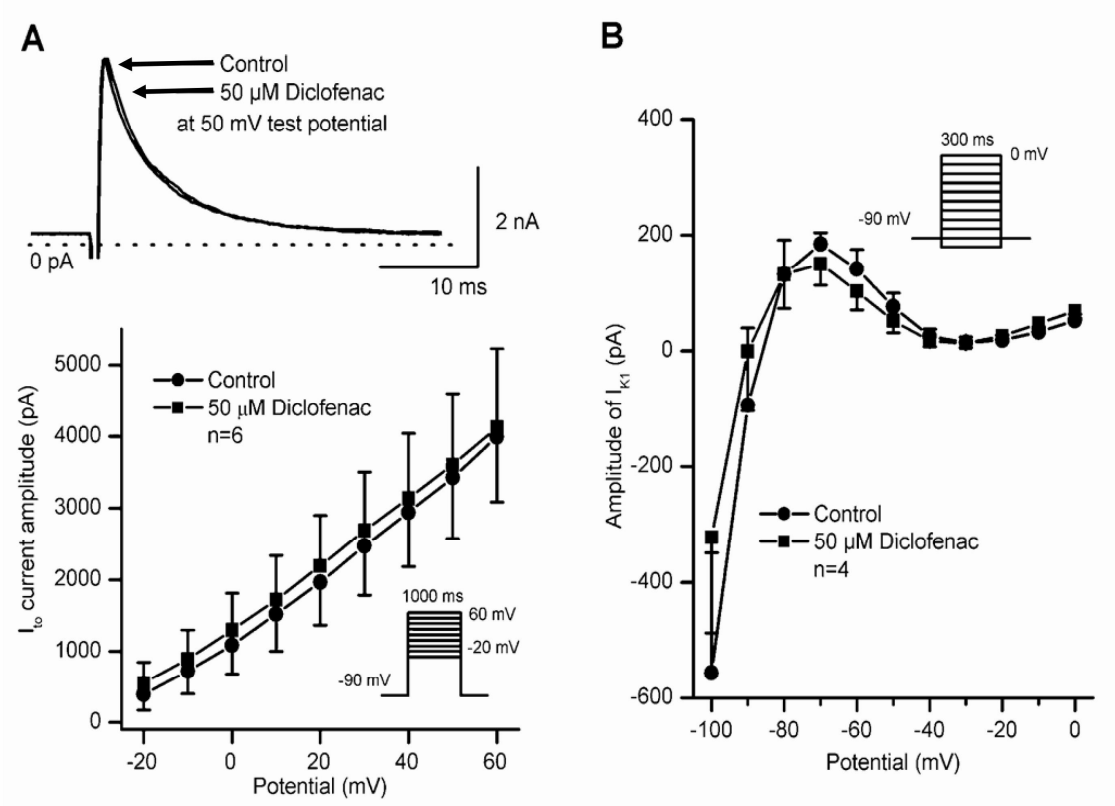

Figure 3. Lack of effect of diclofenac on the transient outward potassium (I_{to}) (A) and on the inward rectifier potassium (I_{K1}) (B) currents in canine ventricular myocytes. A, top: Representative I_{to} current traces under control conditions and after application of 50 µM diclofenac. A, bottom: Current – voltage relationships of I_{to} under control conditions and in the presence of 50 µM diclofenac. Panel B shows steady-state current – voltage relationships of I_{K1} before and after application of 50 µM diclofenac. Insets depict the voltage protocols applied during measurements. Data are expressed as mean ± SEM, n=number of measurements.

and 4B show original I_{Kr} and I_{Ks} current traces in the absence and presence of 30 µM diclofenac, and indicate a significant blockade of I_{Kr} (at 20 mV test potential; from 57.7±5.5 pA to 36.2±2.3 pA, n=5, $p<0.05$) and of I_{Ks} (at 20 mV test potential; from 229.6±15.0 pA to

126.5±10.5 pA, n=6, p<0.05) by diclofenac. The corresponding bottom panels show the current-voltage relationships of I_{Kr} (Fig. 4A) and I_{Ks} (Fig. 4B) before and following superfusion with 30 μM diclofenac.

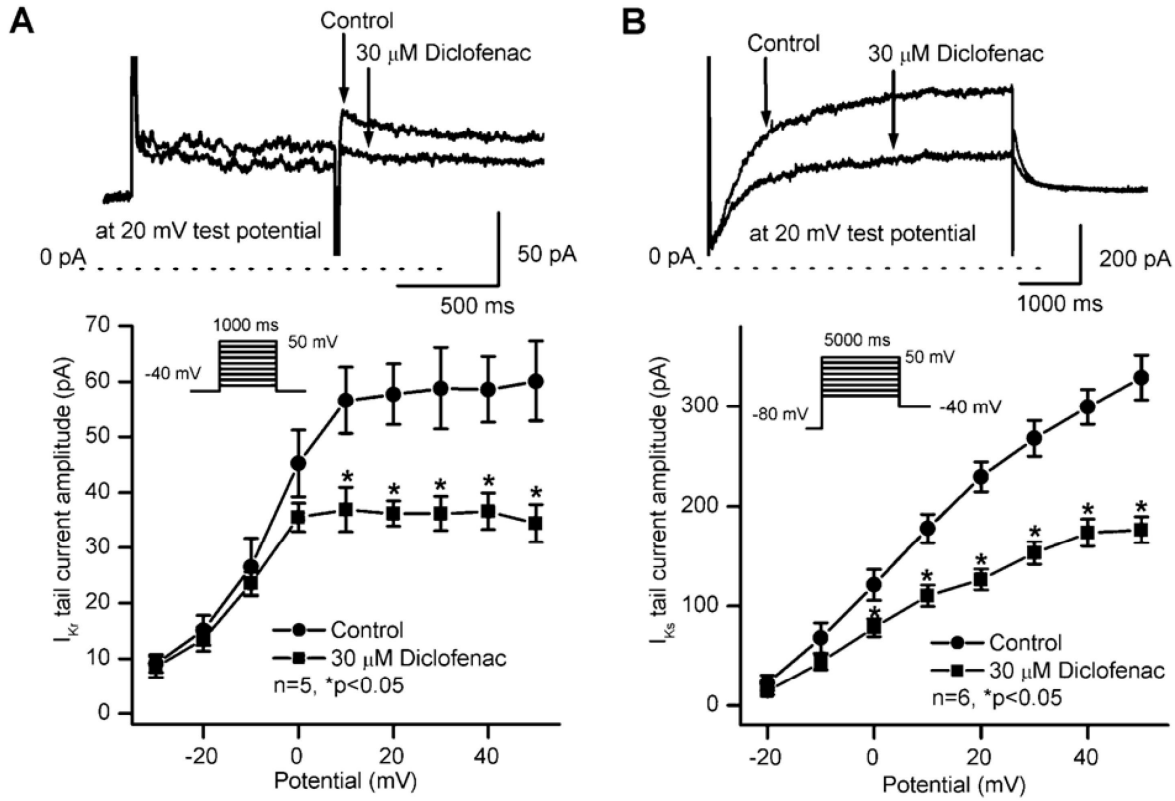

Figure. 4. Effect of diclofenac on the rapid (I_{Kr}) (A) and slow (I_{Ks}) (B) component of the delayed rectifier potassium currents in canine ventricular myocytes. Top panels show representative current traces, bottom panels represent current – voltage relationships under control conditions and in the presence of 30 μM diclofenac. Insets indicate the voltage protocol applied during measurements. Data are expressed as mean ± SEM, n=number of measurements.

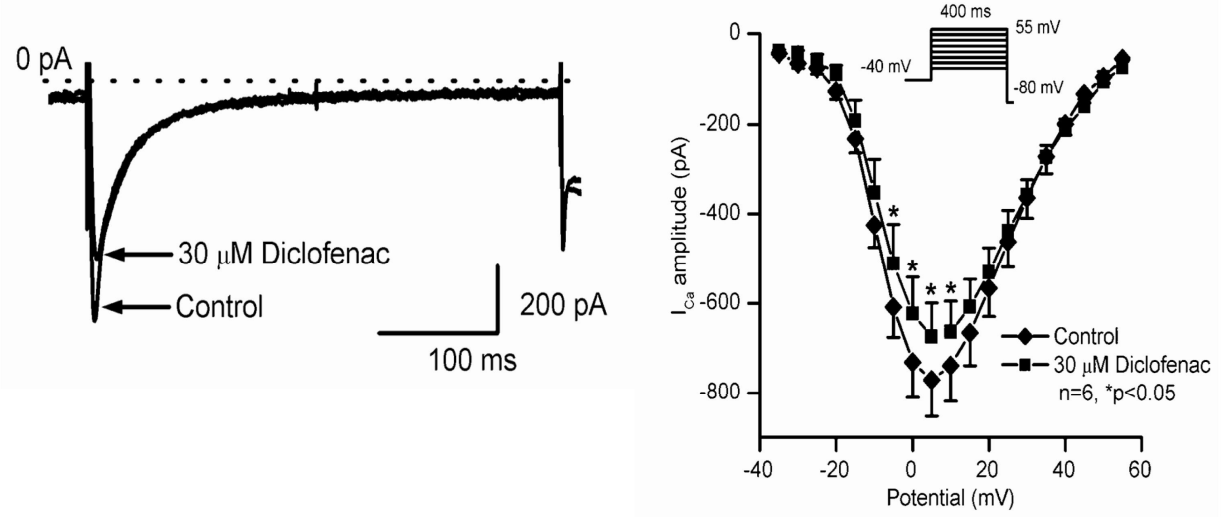

Figure 5. Effect of diclofenac on the L-type calcium current in canine ventricular myocytes. *Left* panel shows representative current traces, *right* panel represents current – voltage relationships under control conditions and in the presence of 30 μM diclofenac. Inset indicates the voltage protocol applied during measurements. Data are expressed as mean ± SEM, n=number of measurements.

I_{Ca} was recorded in the presence 3 mM 4-aminopyridine in order to block I_{to}. The current was evoked by 400 ms-long depolarizing test pulses to voltages between -35 to 55 mV. The holding potential was -80 mV and 75 ms-long prepulse to -40 mV was applied in order to inactivate the sodium current. The pulse frequency was 0.2 Hz. Diclofenac (30 μM) slightly but statistically significantly decreased the amplitude of the current (at 0 mV test potential; from 730.5±79.8 pA to 623.2±82.4 pA, n=6, $p<0.05$) as indicated on Figure 5.

4.2. Analysis of the contribution of I_{to} to repolarization in canine ventricular myocardium

4.2.1. Kinetic properties of I_{to} in canine ventricular cells

I_{to} was activated by 300 ms long depolarizing voltage pulses arising from the holding potential of -80 mV to test potentials gradually increasing up to 50 mV. Earlier results suggested that I_{to} activates and inactivates so rapidly that contributes only to the very early phase of repolarization. However, as illustrated in Fig. 6A-D, inactivation of I_{to} could be fitted as a sum of two exponentials. The fast component had a time constant of less than 5 ms which showed relatively little voltage dependence. Following the rapid initial decay of current a second, much slower component of inactivation was also evident. Its time constant varied between 14 and 23 ms, while its amplitude reached 10-20% of peak current as a function of the membrane potential. This can result in a significant repolarizing force during the plateau phase of the action potential, that is, in a critical period when other membrane currents are relatively week. Specifically, the magnitude of I_{to} was around 32 pA at 50 ms after the initiation of a voltage pulse to 30 mV as calculated using the amplitude and inactivation time constant of the slow phase of I_{to}. For comparison, outward currents of approximately 15 pA and 10 pA are mediated by I_{Kr} and I_{Ks}, respectively, at the same time [22].

Steady-state activation relation for I_{to} was obtained by applying a series of test pulses increasing up to 80 mV in 10 mV steps. The current peaks were divided by the driving force at each membrane potential tested and these ratios were plotted against the respective test potential. When studying steady-state inactivation, test pulses were preceded by a set of 500 ms long prepulses clamped to various voltages between –90 and 20 mV. Peak currents measured after these prepulses were normalized to the peak current measured without prepulse and plotted against the respective prepulse potential. In both cases data were fitted to the two-state Boltzmann function (Fig. 6E-F). Closer inspection of the steady-state activation and inactivation relations obtained for I_{to} shows an overlap between the two curves revealing

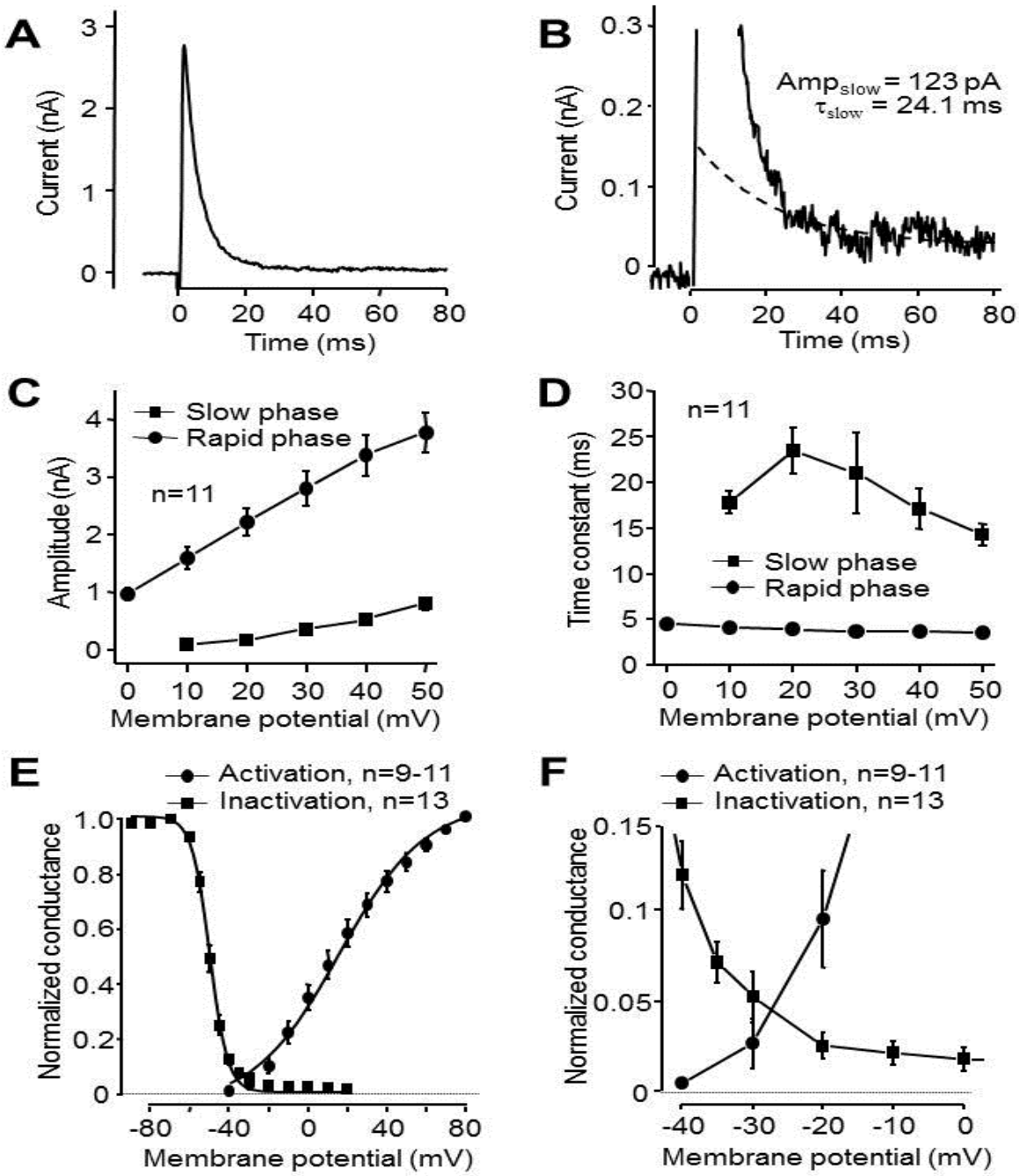

Figure 6. **A**: Representative I_{to} trace recorded at 20 mV from a canine ventricular myocyte. The ordinate was enlarged tenfold in panel **B**, where the dashed line indicates the slowly inactivating component of the current generated by simulation using the estimated amplitude and the time constant values. **C, D**: The decay of I_{to} was fitted as a sum of two exponentials. The estimated amplitudes (**C**) and time constants (**D**) are presented as a function of the membrane potential. **E, F**: Steady-state activation and inactivation curves obtained for I_{to} in canine ventricular myocytes. The overlapping region is enlarged in panel **F**. Data are expressed as mean ± SEM, n=number of measurements.

a "window I_{to} current" similar to those reported earlier for I_{Na} and I_{Ca}. This steady state current, which represents close to 5% of peak I_{to} in the vicinity of -30 mV, may also contribute to repolarization during the late plateau or the early phase of terminal repolarization. In these experiments 1 μM nisoldipine, 0.1 μM dofetilide and 0.5 μM HMR 1556 were always present in the superfusate in order to fully eliminate I_{Ca}, I_{Kr} and I_{Ks}.

Based on data presented in Fig. 6, significant lengthening of APD can be anticipated after suppression of I_{to}. In the absence of a selective blocker, this hypothesis could not be tested so far. However, the following results suggest that the established I_{Ks} blocker, chromanol 293B may be applied for this purpose.

4.2.2. Effect of chromanol 293B on potassium currents

High concentration (100 μM) of chromanol 293B was shown to effectively block I_{Ks} without any effect on inward currents such as I_{Ca} and I_{Na} [74,75]. To study the effect of chromanol 293B on various potassium currents, governing ventricular repolarization in dogs, the compound was applied also at a concentration of 100 μM. In these experiments 1 μM nisoldipine and 0.5 μM HMR 1556 were always added to the control superfusate in order to fully eliminate I_{Ca} and I_{Ks}.

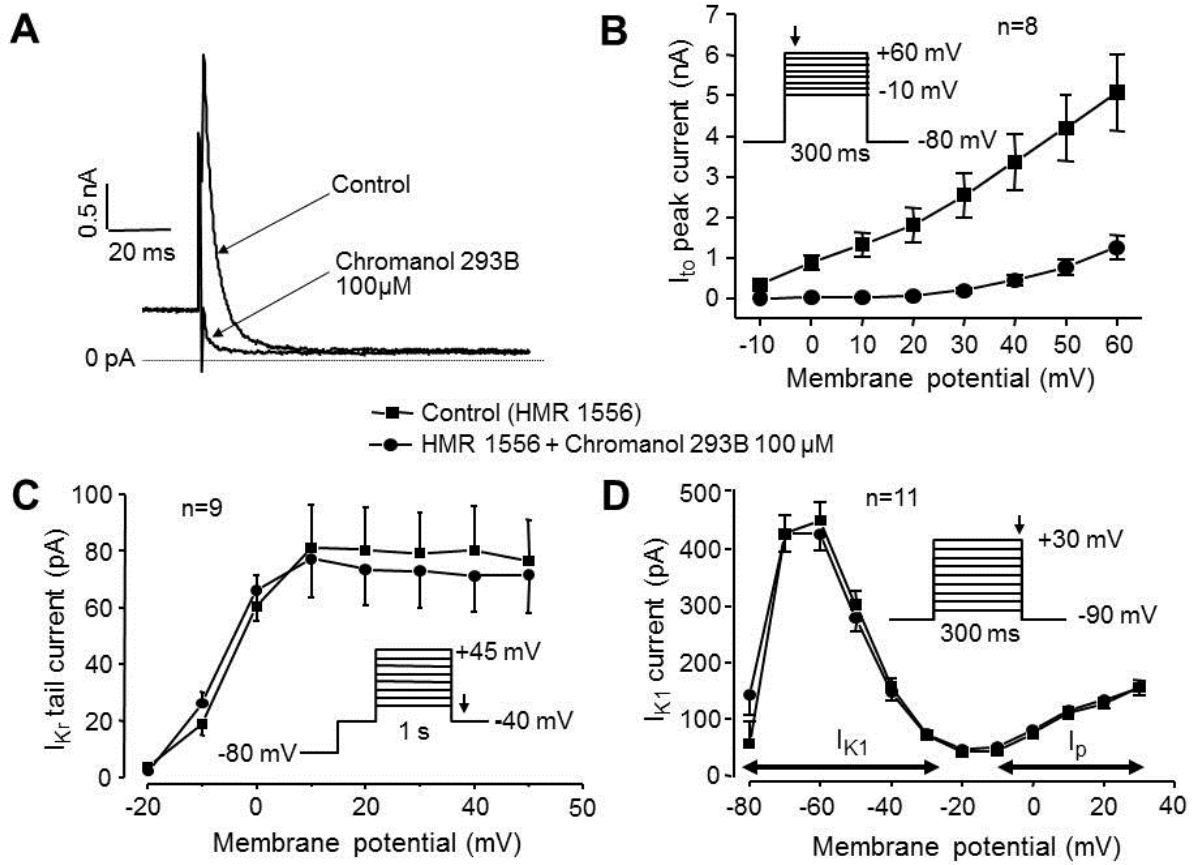

Figure 7. Effects of 100 mM chromanol 293B on I_{to} (A, B), on I_{Kr} tail current (C), and on the steady-state current-voltage relationship obtained at 300 ms (D) in canine ventricular myocytes. The pulse protocols applied are shown in insets. When determining the I-V curve or measuring I_{to} test pulses were delivered at cycle length of 3 s, in case of I_{Kr} measurements the repetition time was 20 s. The 500 ms long prepulse to -40 mV was applied to establish the baseline current when measuring I_{Kr}. Data are expressed as mean ± SEM, n=number of measurements.

As shown in Figs. 7A and 7B, 100 μM chromanol 293B markedly reduced the amplitude of I_{to}. At membrane potentials less than 20 mV block of the peak current was almost complete, while at more positive voltages only fractional. However, chromanol 293B at the same concentration (100 μM) failed to significantly alter I_{Kr} independently of the membrane potential used to activate the current for 1 s (Fig. 7C). Similarly, the drug caused no change in the steady-state current-voltage relationship of the membrane, determinded at the end of 300 ms long pulses clamped to potentials ranging from -80 to 30 mV, i.e. overlapping the voltage range relevant to ventricular repolarization (Fig. 7D).

Within the negative region of this voltage range (between -80 and -30 mV) I_{K1} is active, while in the absence of I_{Ks} the positive branch of the I-V curve is dominated by a mixture of various non-inactivating potassium currents, often called plateau current (I_p). According to results presented in Figs. 7C and 7D, 100 μM chromanol 293B failed to modify repolarizing currents (I_{K1}, I_{Kr}, I_p) other than I_{to} in the presence of HMR 1556.

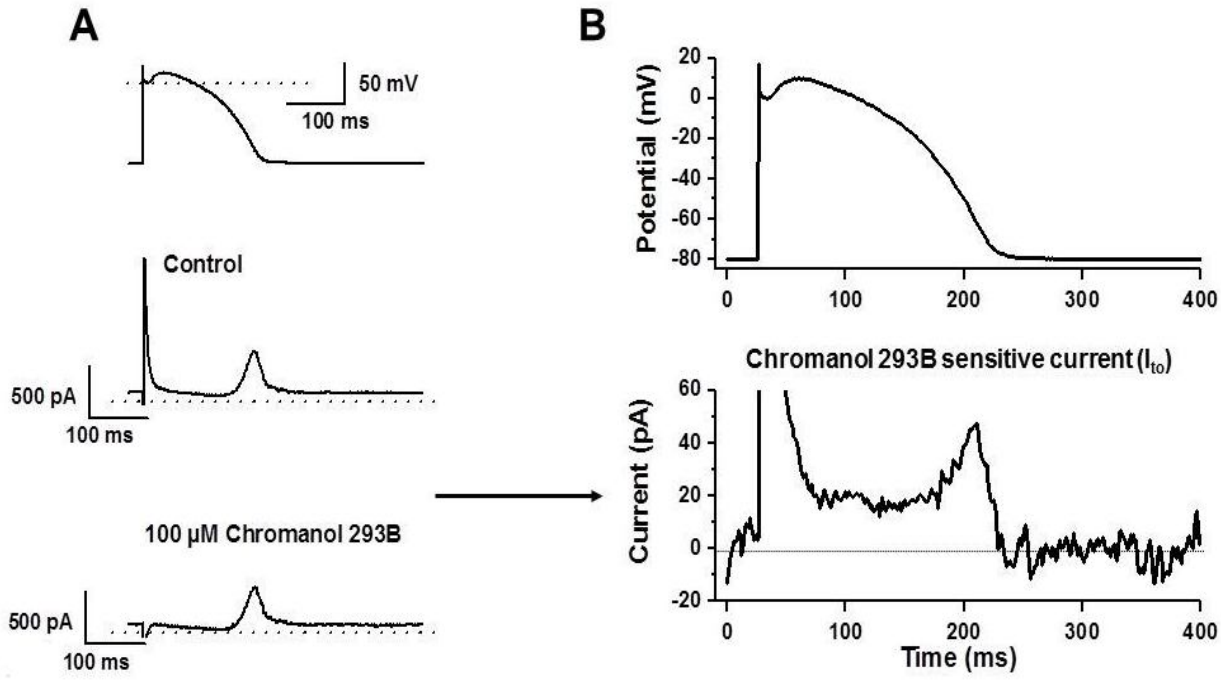

Figure 8. I_{to} current profiles recorded under conventional voltage clamp conditions using a representative action potential as a command pulse. Other ion currents (I_{Ks}, I_{Kr}, and $I_{Ca)}$ were pharmacologically suppressed prior to the experiment. I_{to} was identified as a chromanol-sensitive difference current obtained by subtraction (**B**).

4.2.3. Profile of I_{to} during the action potential

The most relevant way to estimate the profile of any current throughout the time course of the action potential is to apply action potential voltage clamp. This approach, however, requires the use of selective blocker of the current. According to data of literature

[74,75] and results shown in Fig. 7, 100 μM chromanol 293B seems to be a selective blocker of I_{to}, provided the experiment is performed in the continuous presence of an I_{Ks} blocker (0.5 μM HMR 1556).

An action potential waveform, which was previously recorded from a dog left ventricular epicardial slice with sharp microelectrode by conventional microelectrode technique, was delivered as command potential. The other, disturbing ion currents, including I_{Ks}, I_{Kr}, and I_{Ca}, were pharmacologically blocked prior to the measurement. I_{to} was obtained by subtracting the current trace obtained in the presence of 100 μM chromanol 293B from the pre-drug current (Fig. 8).

The resulting chromanol-sensitive current, considered as I_{to}, approximated the current profile expected to be generated during an action potential. At the beginning and during the late plateau this current was consistent with the slowly inactivating phase of I_{to}, which were previously demonstrated in Fig. 6, using conventional square-pulse protocols and with the "window-like" or "late" components of the current.

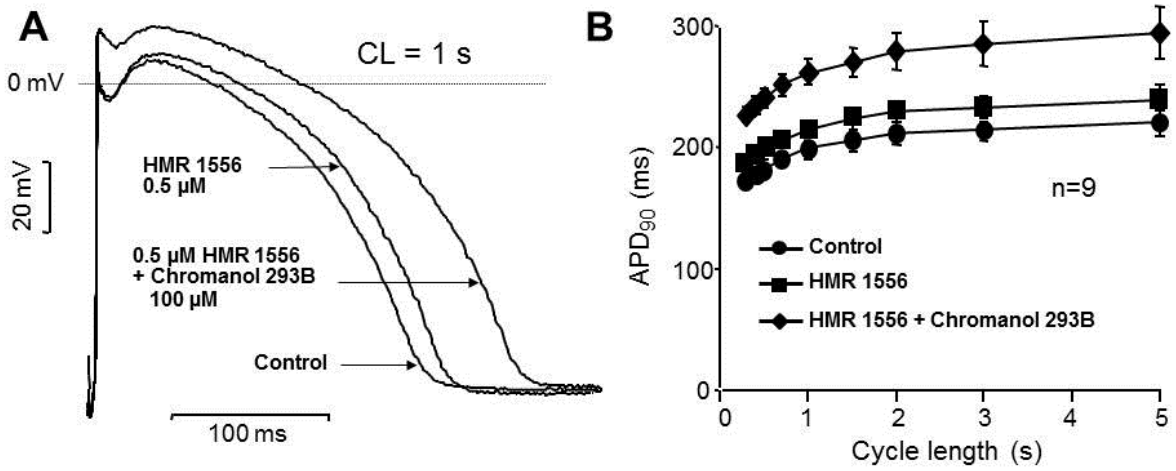

Figure 9. Representative superimposed records demonstrating the effect of 100 μM chromanol 293B on action potential configuration when applied following pretreatment with 0.5 μM HMR 1556 (**A**). **B**: Cycle length dependent changes in action potential duration (APD_{90}) measured under control conditions, following pretreatment with HMR 1556, and in the presence of HMR 1556 plus chromanol 293B. Symbols and bars denote mean ± SEM values obtained in 9 multicellular right ventricular preparations.

4.2.4 Effect of chromanol 293B on action potential configuration

Consequences of I_{to} blockade on action potential configuration were studied in canine right ventricular subepicardial muscle preparations by applying 100 μM chromanol 293B in the presence of 0.5 μM HMR 1556 or 0.1 μM L-735,821 – both drugs providing full I_{Ks} blockade. This latter intervention was necessary to rule out possible changes due to the known

I_{Ks} blocking property of chromanol 293B. As shown in Fig. 9A, full inhibition of I_{Ks} caused only a slight lengthening of APD, as was reported earlier [22]. Additional suppression of I_{to} by administration of 100 μM chromanol 293B in the presence of I_{Ks} blockade significantly lengthened APD and decreased the amplitude of notch following early repolarization.

These changes were accompanied by a marked positive shift of the plateau potential. The APD lengthening effect of I_{to} blockade showed reverse rate-dependent properties, i.e. it was more pronounced at slower than at faster pacing rates (Fig. 9B).

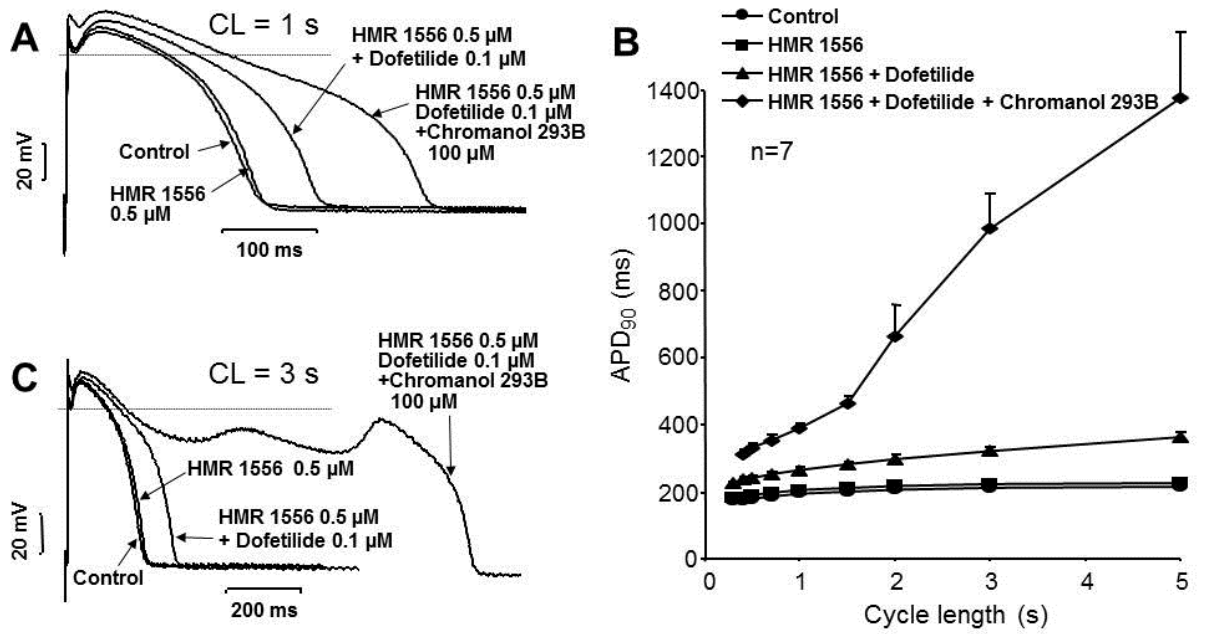

Figure 10. Contribution of I_{to} to repolarization reserve. **A, C**: Representative superimposed action potentials recorded from multicellular right ventricular preparations at cycle lengths of 1 s and 3 s, respectively. In these experiments the preparations were cumulatively treated with HMR 1556, dofetilide, and chromanol 293B (in the above sequence). HMR 1556 was used to prevent the I_{Ks} blocking effect of chromanol 293B, while dofetilide was applied to attenuate the repolarization reserve prior to chromanol 293B superfusion. **B**: Cycle length dependent changes in APD measured under the specified experimental conditions in 7 multicellular right ventricular preparations.

4.2.5. Contribution of I_{to} to repolarization reserve

Repolarization reserve was greatly attenuated by application of 0.1 μM dofetilide for 60 min in the presence of I_{Ks} blockade. Dofetilide markedly lengthened the repolarization of right ventricular muscles by primarily delaying phases 2 and 3, without altering the notch (Fig. 10A). This lengthening of repolarization - as expected - was reversely rate-dependent. After 40 min additional exposure to 100 μM chromanol 293B the prolongation of repolarization dramatically increased, especially at slow stimulation rates, also in a reversely rate-dependent manner. The magnitude of the chromanol-induced lengthening of APD at slow rates was greater than the arithmetical sum of the APD lengthening caused by chromanol 293B and dofetilide alone (Fig. 10B). In addition, in 5 out of 7 experiments early

afterdepolarizations (EADs) were observed at cycle lengths longer than 2 s (Fig. 10C). EADs have never been observed under these experimental conditions when in the presence of HMR 1556 dofetilide or chromanol 293B were applied alone.

4.3. Effects of diabetes on ventricular repolarization and the underlying transmembrane potassium currents in rabbit hearts

4.3.1. Effect of diabetes on ventricular repolarization

Fig. 11a shows representative ECG recordings obtained from control and diabetic rabbits. After 3 weeks of alloxan treatment the QT interval of diabetic animals was slightly, but significantly longer compared to that measured before alloxan administration (134±2.4 ms, n=9 and 123±2.7 ms, n=10, respectively, $p<0.05$). Since the RR intervals were also increased in the diabetic rabbits (195±4.9 ms *versus* 182±4.5 ms, $p<0.05$, n=9-10), the diabetes-induced lengthening of the QT_c interval was less pronounced (from 145±2.8 ms to 155.2±1.8 ms), however, it was statistically significant ($p<0.05$, n=9 and 10, respectively), as shown in Fig. 11b. No significant change was observed in the control animals during the identical period of 3 weeks.

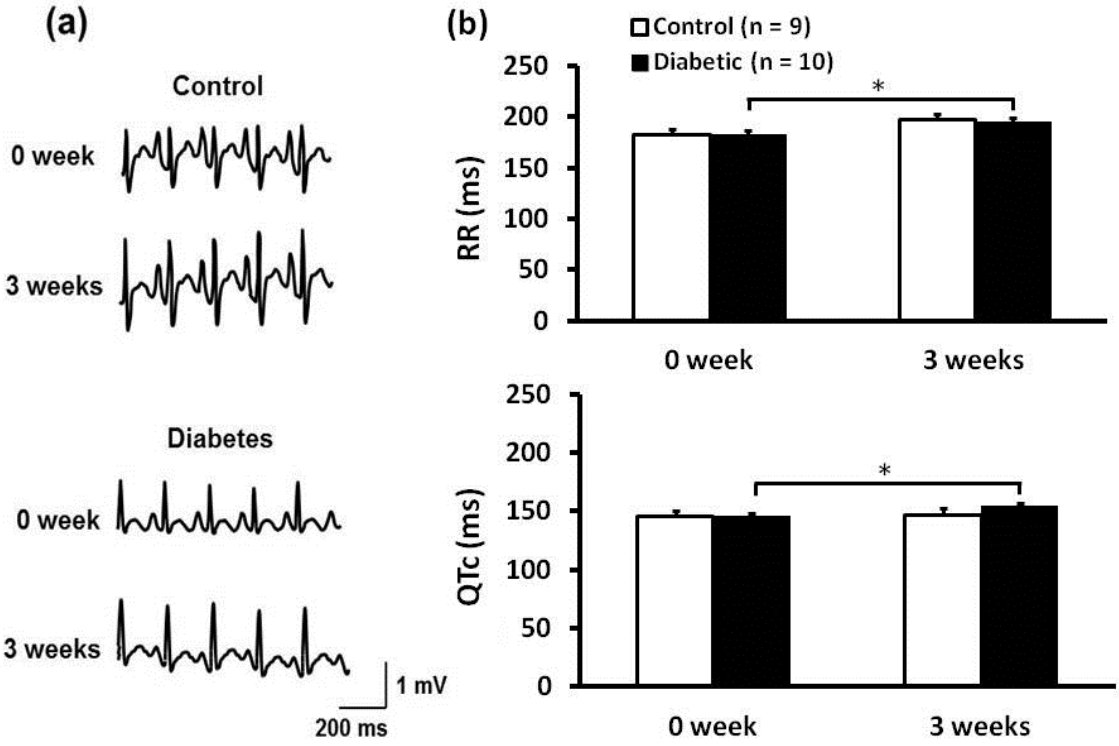

Figure 11. Representative ECG recordings (b) obtained at the beginning (0 week) and end (3 weeks) of the experiment in non-diabetic control and diabetic animals.

4.3.2. Diabetes-induced changes in ion currents

Steady-state current-voltage relationship of the membrane was determined by applying 300 ms long voltage pulses to test potentials ranging from -120 mV to 0 mV, arising from the holding potential of -90 mV. Membrane currents measured at the end of these pulses were plotted against their respective test potentials.

As shown in Fig. 12, no significant differences were observed in the steady-state current-voltage relationship, and consequently, in the density of I_{K1} (at -100 mV current amplitude which was -12.82±0.94 pA/pF for control and -12.64±0.89 pA/pF for diabetic rabbits, n=35 and 40, respectively, N.S.), in ventricular myocytes obtained from control and diabetic rabbits.

I_{to} was evoked by applying 400 ms long test depolarizations to voltages ranging from -10 to 60 mV with 3 s interpulse intervals. The holding potential was -90 mV. The amplitude of I_{to} was defined at each membrane potential as a difference of the peak outward current measured at the beginning and the steady-state current at the end of the test pulse.

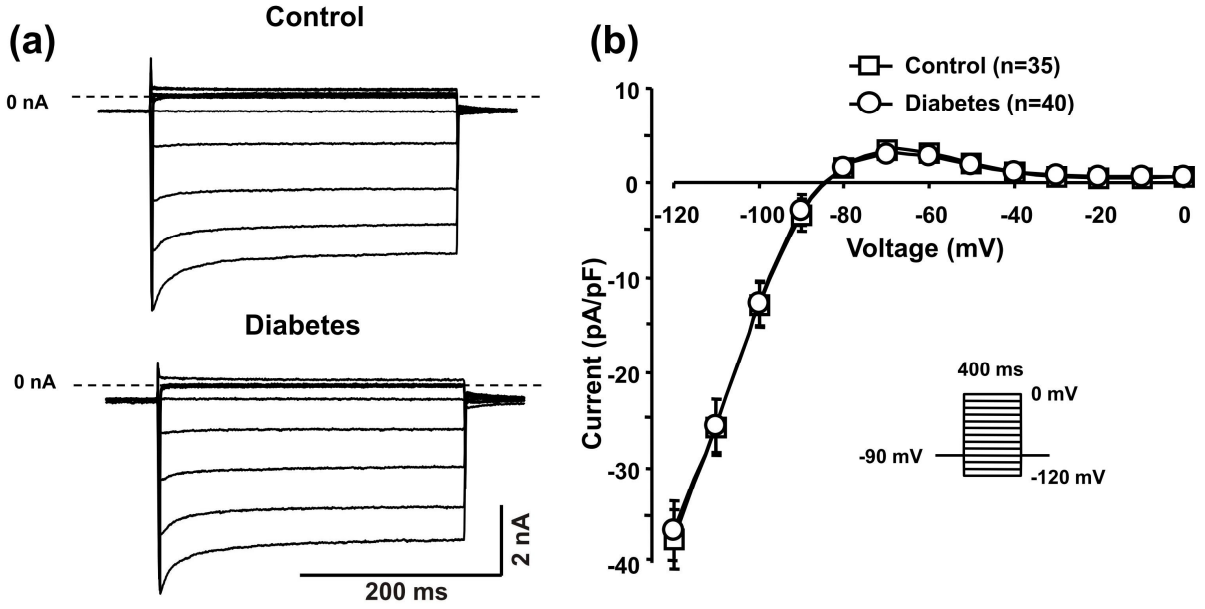

Figure. 12. Current-voltage (I-V) relationship of the inward rectifier potassium current (I_{K1}) in rabbit ventricular myocytes isolated from healthy and diabetic animals. (**a**). Representative I_{K1} families. **(b)**. Average results are presented as I-V curves. The applied voltage protocol is shown in the inset. Dashed lines indicate the zero current level. Symbols and bars are mean values ± SEM, n denotes the number of cells tested.

The amplitude of I_{to} did not differ significantly in ventricular myocytes isolated from control and diabetic rabbits (at 50 mV the current amplitude was 5.63±0.44 pA/pF in control *versus* 5.46±0.31 in diabetic rabbits, n=30 and 38, respectively, N.S.), as shown in Fig. 13. The kinetics of recovery from inactivation was also not altered by diabetes (the time constant

was 1080±248 ms in control vs. 1110±232 ms in diabetic rabbits, n = 30 and 38, respectively, N.S.).

I_{Kr} was activated by 1 s long depolarizing test pulses to membrane potentials ranging from -20 mV to 50 mV at the frequency of 0.05 Hz. 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 30 μM chromanol 293B (gift from Sanofi-Aventis Pharma, Frankfurt, Germany) in order to eliminate I_{Ks}. Results displayed in Fig. 14 indicate that the amplitude of I_{Kr} (at 30 mV the current amplitude was 0.64±0.02 pA/pF in control *versus* 0.63±0.03 pA/pF in diabetic rabbits, n=19 and 21, respectively, N.S.), was not altered in the alloxan-induced diabetes.

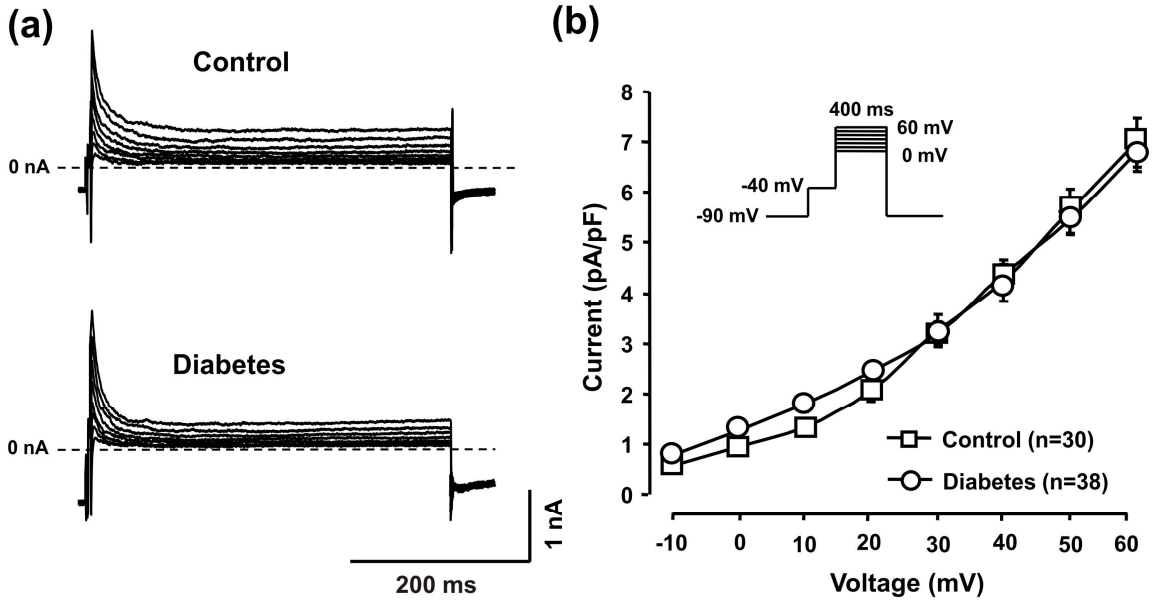

Figure 13. Current-voltage (I-V) relationship of the transient outward potassium current (I_{to}) in rabbit ventricular myocytes isolated from healthy and diabetic animals. (**a**). Representative I_{to} families. **(b)**. Average results presented as I-V curves. The applied voltage protocol is shown in the inset. Dashed lines indicate the zero current level. Symbols and bars are mean values ± SEM, n denotes the number of cells tested.

Activation kinetics of I_{Kr} measured using the envelope tail test protocol [22], was not altered by diabetes. At 30 mV the activation time constants for I_{Kr} were 35.5±3.12 ms in control versus 32.5±2.65 ms in diabetic rabbits (n=19 and 21, respectively, N.S.). The deactivation kinetics was studied at -40 mV by fitting with two exponentials the deactivating "tail current" at -40 mV. The deactivation of the I_{Kr} was also unaffected by diabetes, the corresponding time constants were τ_1=650.8±33.4 ms and τ_2=6636±395 ms in control rabbits *versus* τ_1=580.1±25.4 ms and τ_2=5953±364 ms (n=19 and 21, respectively, N.S.).

I_{Ks} was activated by depolarizing test pulses of 5 s duration clamped to membrane potentials ranging between -20 mV and 50 mV and applied at frequency of 0.1 Hz. The amplitude of the tail current measured upon repolarization to the holding potential of -40 mV was plotted against the activation voltage and was used to define the magnitude of I_{Ks}.

These experiments were performed in the presence 1 μM E-4031 (gift from Institute for Drug research, Budapest, Hungary) in order to eliminate I_{Kr}. As displayed in Fig. 15, the amplitude of I_{Ks} was significantly less in diabetic than in non-diabetic rabbits at 50 mV (0.86±0.17 pA/pF *versus* 1.48±0.35 pA/pF, $p<0.05$, n=21 and 19, respectively. Activation kinetics of I_{Ks} measured using the envelope tail test protocol was not altered by diabetes. At 50 mV the activation time constants for I_{Ks} were 888.5±43.12 ms in control *versus* 798.15±32.65 ms in diabetic rabbits (n=19 and 21, respectively, N.S.). The deactivation kinetics was studied at -40 mV by fitting with a monoexponential function the deactivating tail current at -40 mV. The deactivation of the I_{Ks} was unaffected by diabetes, the corresponding time constants were τ=167.8±9.4 ms in control rabbits *versus* τ=159.1±15.4 ms (n=19 and 21, respectively, N.S.).

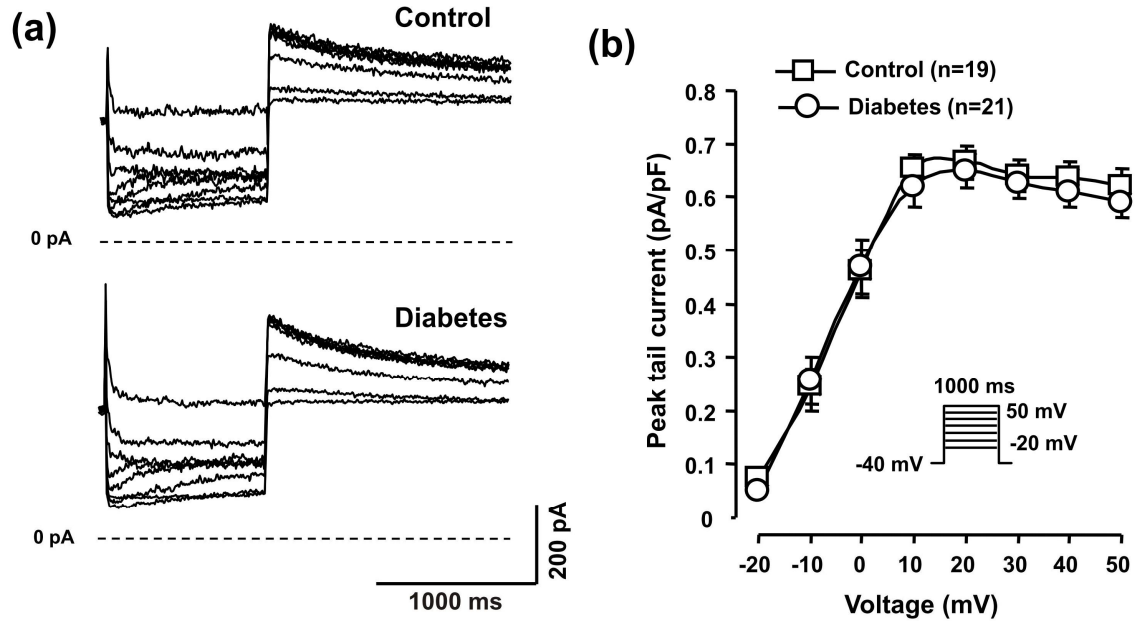

Figure 14. Amplitude of the rapid component of the delayed rectifier potassium current (I_{Kr}) in myocytes derived from diabetic and healthy rabbit hearts. **(a)**. Current families of the fully activated I_{Kr} currents followed by tail currents upon returning to the holding potential of -40 mV. (**b**). The voltage dependence of activation of I_{Kr} generated by plotting the mean amplitudes of the I_{Kr} tail currents against the activation voltage. Symbols and bars are mean values ± SEM, n denotes the number of cells.

L-type inward calcium current (I_{CaL}) was recorded in the presence 3 mM 4-aminopyridine in order to block I_{to}. The current was evoked by 300 ms long depolarizing test pulses to voltages between -40 to 55 mV, arising from the holding potential of -40 mV. Peak

values of I_{CaL} were plotted against the respective test potentials (Fig. 16). The density of I_{CaL} was not significantly different in the control and diabetic groups (at 5 mV the current amplitude was -6.22±0.32 pA/pF in control *versus* -6.23±0.33 pA/pF in diabetic rabbits, n = 22 and 24, respectively, N.S.), having also similar voltage-dependence of activation.

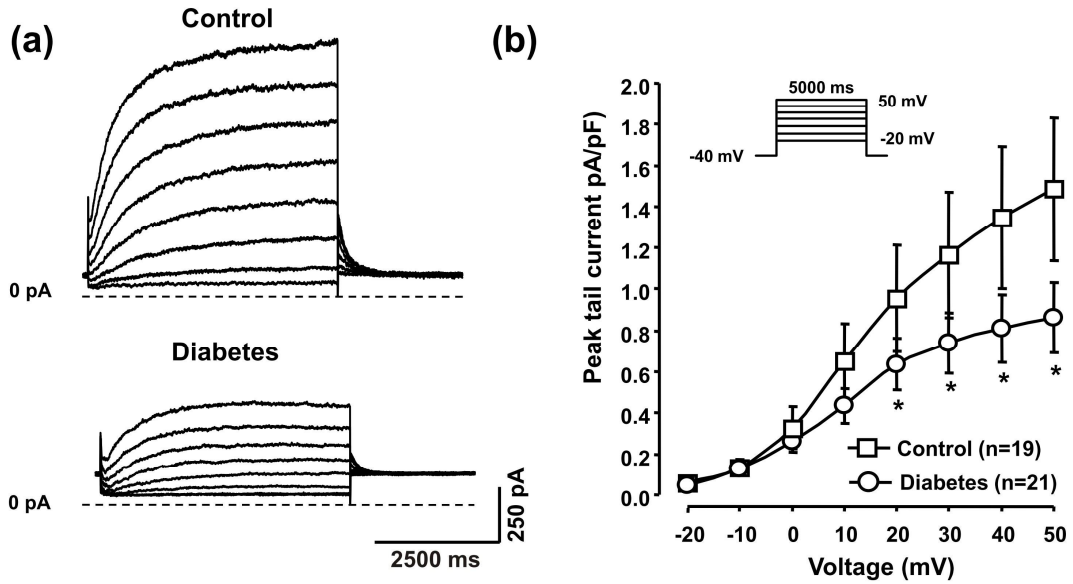

Figure 15. Diabetes-induced changes in the slow delayed rectifier potassium current (I_{Ks}) in rabbit cardiomyocytes. **(a)**. Current families of the fully activated I_{Ks} currents followed by tail currents upon returning to the holding potential of -40 mV. **(b)**. Voltage dependence of activation of I_{Ks} current was generated by plotting the mean amplitudes of the I_{Ks} tail currents against the activation voltage. Symbols and bars are mean values ± SEM, n indicates the number of cells, asterisks denote significant differences ($p<0.05$) from control.

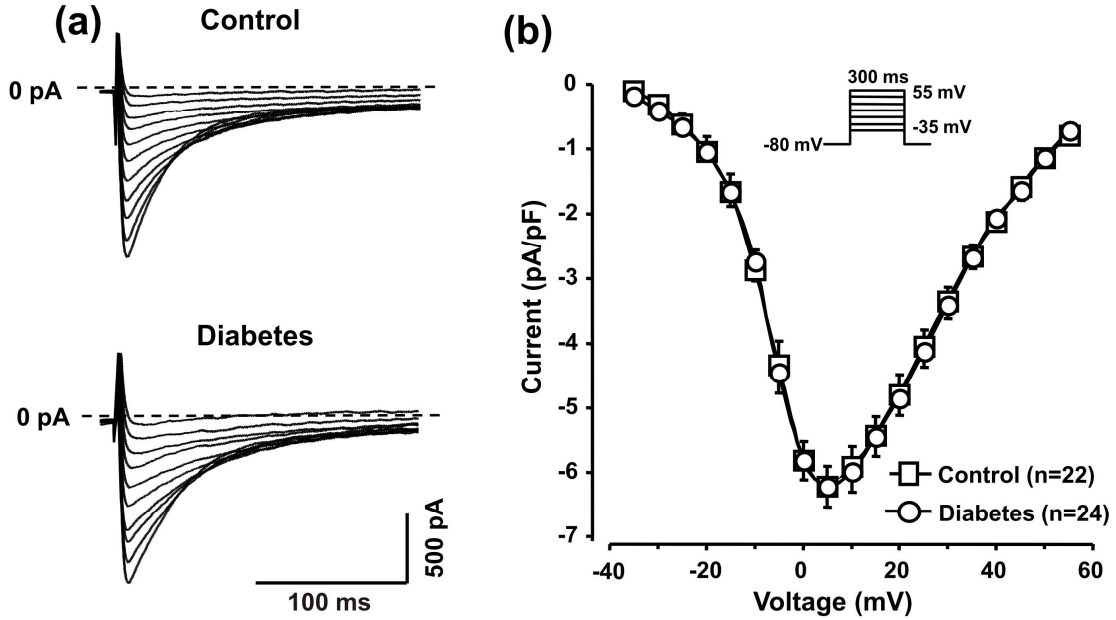

Figure 16. Current–voltage (I–V) relationship of the L-type inward calcium current (I_{CaL}) in rabbit ventricular myocytes isolated from healthy and diabetic animals. (**a**) Representative I_{CaL} families. (**b**) Average results are presented as I–V curves. The applied voltage protocol is shown in the inset. Dashed lines indicate the zero current level. Symbols and bars are mean values ±SEM, n denotes the number of cells tested.

5. DISCUSSION

5.1. The effect of NSAID drug diclofenac on cardiac repolarization in dog ventricular myocytes.

The main results of this study show that in the normal heart, diclofenac does not exert marked cardiac electrophysiological effects and does not enhance risk of arrhythmia, however, in hearts where repolarization reserve is impaired, its moderate inhibition of I_{Ks} and I_{Kr} may lead to prolongation of ventricular repolarization and may also increase proarrhythmic risk.

Our results indicate that under normal conditions diclofenac exhibits minor effects on the transmembrane ion currents in canine ventricular myocytes, inhibiting I_{Kr}, I_{Ks} and I_{Ca} currents but leaving I_{to} and I_{K1} unchanged. Only a slight action potential lengthening was induced in ventricular muscle preparations and the drug shortened the action potential duration in Purkinje fibers. The maximum upstroke velocity was decreased in both preparations by diclofenac. However, larger repolarization prolongation was observed in preparations with impaired repolarization reserve.

Little is known about the direct cardiac electrophysiological effects and the possible proarrhythmic potency of diclofenac. Most of the information about the action of this drug on ion currents arises from measurements in non-cardiac cells. It was reported that diclofenac activated the transient outward K^+ current [76] and inhibited sodium current in rat cerebellar granule cells [77] and in myoblasts [78]. It was also observed that diclofenac enhanced KCNQ2/Q3 currents [79] while others recently reported that the drug served as an activator of KCNQ4 and a blocker of KCNQ5 channels [80]. The only ionic current data obtained in ventricular cells were measured by Yarishkin *et al.* [81], who described inhibition of L-type Ca^{2+} current by the drug in neonatal rat ventricular myocytes, however, in rat aortic smooth muscle cells diclofenac did not influence this current [82].

Our results clearly showed that diclofenac did not influence I_{to} and I_{K1} currents even at high concentration but decreased the amplitude of I_{Kr} and I_{Ks} currents in canine ventricular myocytes. In spite of the significant I_{Kr} blockade by the drug just a small but statistically significant action potential lengthening was detected following diclofenac administration. Some of our other observations may explain these seemingly conflicting results. Diclofenac significantly decreased the maximum upstroke velocity in canine ventricular muscle and also in Purkinje fibers, and shortened the action potential duration in Purkinje fibers. These results indicate the Na^+ channel blocking property of the drug. It is well established that the late or

persistent component of the Na^+ current contributes to the action potential plateau, which is most significant in Purkinje fiber [83]. Therefore, blockade of this current tends to limit the action potential prolongation resulting from the I_{Kr} inhibition by diclofenac. Indeed, a similar reduction of the action potential duration prolonging effect by additional $I_{Ca,L}$ inhibition was demonstrated earlier in the case of the neuroleptic risperidone that blocks I_{Kr} [84]. Therefore, the slight decrease of L-type Ca^{2+} current by high concentration of diclofenac found in this study may also help to counteract the action potential lengthening effect of I_{Kr} blockade. I_{Ks} blockade caused by diclofenac might only marginally influence action potential duration but attenuates repolarization reserve [85,22,23].

These results imply that diclofenac may not augment spatial repolarization heterogeneity. The influence of diclofenac on action potential repolarization was also investigated after impairment of the repolarization reserve of the preparation by adding 30 μM $BaCl_2$, which partially blocks I_{K1} current. The drug induced a marked action potential lengthening, i.e. the APD lengthening effect of diclofenac was significantly larger in these conditions than found in ventricular preparations with normal repolarization reserve.

Repolarization reserve can be reduced by several congenital or acquired pathophysiological conditions such as genetic defects affecting ion channels, diabetes mellitus, hypothyroidism, heart failure etc., including reversible cardiac hyperthrophy (athlete's heart). Animal and human experimental data indicates that the downregulation or dysfunction of the I_{Ks} current plays a critical role in the development of cardiac repolarization reserve impairment [86]. In competitive athletes, slight impairment of repolarization reserve [87] does not result in a significant risk of arrhythmia but together with additional factors, otherwise considered as mild ones (such as increased sympathetic tone, seemingly harmless medications, doping agents, dietary constituents, hypokalemia due to intense sweating, and early and undiagnosed cardiomyopathy or other pathological anomalies), these hits on repolarization may add up and can cause repolarization abnormalities occasionally leading to sudden cardiac death. In this regard, earlier it was found that the beat-to-beat variability of the QT interval, a novel ECG parameter characterizing temporal instability of cardiac repolarization [88], was higher in professional soccer players compared to their age-matched controls with no significant sports activities [89].

The applied concentration of diclofenac in the present study was somewhat higher than the reported therapeutic level, which is approximately 2 – 7 μM/L based on the therapeutic blood level data after 50 mg oral administration diclofenac [90, 91]. It is well known that top athletes widely use NSAIDs, to treat their sports injuries frequently

diclofenac, in larger doses that can result in significantly higher plasma and tissue levels than considered therapeutic. Also, top athletes may sometimes take diclofenac to eliminate sports injury related pain in much larger doses than recommended to return to competition as soon as possible even prior to full recovery.

It is important to emphasize that based on our results, application of diclofenac alone, even in high doses, probably does not increase the risk of arrhythmias. Therefore, individuals taking diclofenac under proper medical control should not be concerned about proarrhythmic side effects. However, diclofenac administration may add to the increased risk for serious arrhythmia development in persons associated with subsidiary risk factors including certain diseases or genetic defects that impair repolarization, as well as in individuals taking part in top competitive sports activities.

5.2. Role of I_{to} in the repolarization of canine ventricular myocardium

The main finding and the message of this study is that I_{to} is involved in governing repolarization and, as a consequence, it contributes significantly to the repolarization reserve. Accordingly, inhibition of I_{to} in the presence of impaired repolarization reserve may elicit excessive repolarization lengthening resulting in EAD formation with the concomitantly enhanced proarrhythmic risk.

Our present experiments showed that the inactivation of I_{to} is a double exponential process, though there are many reports that approximate decay of I_{to} with a single exponential function [92,93,94]. The most likely reason that these studies found only a single component of inactivation for I_{to} is that the majority of these experiments were performed at room temperature. In addition, the amplitude of the second slow component is much smaller than the fast one, thus it can be easily overlooked in the nA scale. Several studies, however, reported a rapid and a slow phase of I_{to} inactivation at 36 – 37°C. The inactivation time constants found in our work are similar described in these studies [95,96].

Our other important finding is that I_{to} shows a small overlap between the steady-state activation and inactivation curves revealing a “window I_{to} current” similar to those reported earlier for I_{Na} and I_{Ca}. However, in several previous studies the existence of such “window current” was not reported and the lack of effect of chromanol 293B on steady-state current in this “window potential” range may raise question regarding to the “window I_{to} current” theory.

The possible explanation of this controversy can be due to the unexplored and complicated nature of the gating mechanism of K_v4 channels, which are most likely the pore forming alpha subunit of dog I_{to}. Several studies showed that the complexity of gating in K_v4 channels is explained by models with multiple inactivation components accounting for both open- and closed-state inactivation. Upon reaching the open state, it is supposed that inactivation can proceed through either a closed-state mechanism or an open-state mechanism. Those several gating models published in the literature agree on the predominance of closed-state inactivation in K_v4 channel gating [97]. However, during recovery from open-state inactivation channels must reopen before reaching closed state. On the basis of these models we can predict reopening of K_v4 channels upon membrane repolarization, which is actually demonstrated Gebauer *et al*. [98] with $K_v4.2$ channels. This theory could be one possible explanation of the "late" I_{to} current found in this study.

The influence of I_{to} on cardiac action potential duration is not well understood in canine ventricle. The general belief is that I_{to} contributes only to early (phase 1) repolarization so it fails to directly influence APD, or it may alter APD indirectly by shifting the activation, deactivation, or inactivation of other plateau currents. The impact of pharmacological block by 4-aminopyridine, the commonly used I_{to} inhibitor, on ventricular APD is controversial. Both moderate shortening and lengthening of APD were reported [99,100,101,102] with 4-aminopyridine - depending on the concentration, species, region of myocardium, or experimental conditions. The major problem with 4-aminopyridine in action potential measurements is the lack of selectivity. Millimolar concentrations of 4-aminopyridine effectively suppressed I_{Kr} in addition to inhibition of I_{to}. Another previous report suggested inhibitory effect of 4-aminopyridine on I_{K1} [103]. These results greatly limit the value of 4-aminopyridine in action potential measurements indicating that data with 4-aminopyridine on cardiac repolarization should be interpreted with caution, and a more selective blocker of I_{to} (e.g. chromanol 293B in the presence of full I_{Ks} blockade) should be used to study the role of I_{to} in repolarization.

Lengthening of repolarization induced by 30 μM chromanol 293B in the presence of I_{Kr} blockade has been previously reported in dog ventricular muscle [104] which was attributed to combined inhibition of I_{Ks} and I_{Kr}, however, the possible contribution of an I_{to} blockade was not considered. Present results suggest that this lengthening of repolarization could rather be attributed to the inhibition of I_{to}.

Our conclusion was based on the assumption that chromanol 293B, in addition to blocking I_{Ks}, exerts its effect on cardiac repolarization by inhibiting exclusively I_{to}. Although

in the present study we carefully examined and ruled out the possible effects of chromanol 293B on other major transmembrane currents, such as I_{Kr}, I_{K1}, I_p, I_{Ca} and late I_{Na}, we can not completely rule out the possible effects of chromanol 293B on all ion currents that may theoretically influence ventricular repolarization (for example calcium-dependent chloride current). It is also well known that there are significant transmural and regional differences in the expression of I_{to}. In our study we used right ventricular subepicardial muscle preparations to study the effect of I_{to} inhibition on repolarization, since this preparation was reported to strongly express I_{to}. Due to the existing transmural and regional inhomogeneity of I_{to} expression, inhibition of I_{to} may induce effects of different magnitudes in the various regions of the ventricular wall, which may further enhance the dispersion of repolarization, and as such is expected to increase the substrate for proarrhythmic risk.

As a major result, we described in dog ventricular muscle that the voltage-dependent I_{to} has a second component with slow inactivation kinetics and exhibit late activation. These direct effects of I_{to}, in addition to the previously recognized indirect effects on I_{Kr} and I_{Ca} caused by voltage changes, suggest substantial contribution of I_{to} to repolarization during the plateau and terminal phase of repolarization. Thus, inhibition of I_{to} causes a positive shift of plateau voltage combined with lengthening of the overall repolarization when studied in the presence of full I_{Ks} blockade. More importantly, the lengthening of APD when repolarization reserve is impaired - under conditions of combined I_{Kr} and I_{to} blockade - may be so excessive that it may result in generation of EADs. Since I_{to} is down-regulated in many diseases including heart failure, and because most of the drugs available in the therapy have never been tested carefully for a possible inhibitory effect on I_{to}, the findings of the present study have important therapeutical and safety pharmacological implications regarding the risk of drug induced QT prolongations and the related life threatening arrhythmias.

5.3. Role of slow delayed rectifier K^+-current in the repolarization in a diabetic rabbit heart.

The major finding of this study is that experimentally induced type 1 diabetes mellitus caused only a moderate but statistically significant lengthening of the QT_c interval in the rabbit heart, which was associated with a marked reduction in the density of I_{Ks}. No change in other ion currents (I_{Kr}, I_{to}, I_{CaL} and I_{K1}) was observed.

The result that I_{to} current was not suppressed in rabbit heart by the diabetes was an unexpected finding. Several studies in the rat showed that experimentally induced type 1

diabetes mellitus lengthened cardiac action potential duration and decreased the amplitude of I_{to} [56,57,58,59,70,71]. Also, in our previous study in the dog both I_{to} and Kv 4.3 protein, which is the dominant pore-forming subunit in canine and also in human heart were found to be downregulated [60]. In this study in the rabbit we did not observe significant changes in I_{to} current. The reason for this discrepancy can be best explained by the different molecular basis of rabbit I_{to}. In rabbits, unlike in rats, dogs and humans, I_{to} is mediated mostly by Kv 1.4 and not by Kv 4.3 channels [105,106].

The role of I_{Ks} in normal repolarization (*i.e.* in healthy individuals) was questioned. The reduction of I_{Ks} current alone is not expected to cause a marked APD lengthening and strong QT_c interval prolongation, unless sympathetic tone is increased [22,23,107]. Here, we report that diabetes caused only a moderate (about 6.9 %) QT_c lengthening effect. It has been reported that in rabbit I_{Ks} is a larger current than in dog and human [85] , therefore I_{Ks} reduction in principle may cause moderate (<7 %) but statistically significant APD/QT_c prolongation, especially in intact animals, which may have normal or even enhanced sympathetic tone.

The mechanism by which diabetes mellitus can influence expression of cardiac ion channels is not fully understood. Only some diabetes induced effects on I_{to} were reported. For example activation of the renin-angiotensin system was demonstrated in insulin-dependent diabetic rats. It was also reported that the increased level of angiotensin II attenuated I_{to} [71]. It was found that inhibition of the formation or action of angiotensin II reversed the attenuated I_{to} in both type 1 and type 2 diabetes. This observation can be explained by the fact that angiotensin II has numerous and diverse cellular effects mediated by protein kinase A, protein kinase C, and tyrosine kinases, which may be linked to the inhibition of certain transmembrane ion channels as I_{to} and sustained outward current (that is common mainly in rat) [108]. The mechanism of the reduction of I_{Ks} by diabetes was not studied.

It is to be emphasized that in our study I_{Ks} was the only ion current which was affected by the diabetic changes. This result seems to be different from the data of Wang and his co-workers, who very recently published some new results regarding the effect of alloxan-induced electrophysiological effects in rabbit hearts. They reported significant reduction of I_{Kr} and HERG in alloxan-induced diabetes of the rabbit. In accordance with this, lengthening of the QTc interval was much more pronounced in these studies [61,62]. Since 11 weeks of diabetes were monitored in those reports - comparing with the 3 weeks diabetic period of our rabbits, it is possible that the diabetes-induced changes in various ion currents can develop on different time scales.

Although I_{Ks} plays a minor role in the repolarization of the normal heart (*i.e.* in healthy individuals), its importance under certain pathophysiological conditions seems to be increased [22,75,107,109,110]. According to the concept of "*repolarization reserve*" [11,111] K^+ currents may compensate for each other to secure the repolarization process [11,23,111,112]. Therefore, under normal conditions block of one type of K^+ current causes not necessarily an excessive lengthening of action potential duration, since other types of K^+ currents may provide sufficient repolarization. If repolarization is excessively lengthened due to any reasons (such as blockade of I_{Kr}, hypokalaemia, genetic abnormality, or bradycardia), the subsequent increase in action potential duration would facilitate the activation of I_{Ks} and provide a negative feedback mechanism to limit further lengthening [22,23,112,113]. In the case of inherited (*e.g.* long QT syndromes) or acquired disease (*e.g.* ion channel remodelling caused by heart failure, acute myocardial infarction, or diabetes mellitus) deficiency of this mechanism may occur, which may lead to excessive prolongation of repolarization and increased propensity for development of early afterdepolarizations in response to a relatively weak inhibition of a K^+ current [114,115,116,117,118]. Therefore, since I_{Ks} is an important contributor to the repolarization reserve, reduction of the density of this current in diabetes mellitus would increase the proarrhythmic risk, especially when another repolarizing potassium current is also diminished (e.g. due to a genetic defect of a K^+ channel, or in the case of acquired long QT syndrome). Consequently, diabetic patients may carry an increased proarrhythmic risk due to their compromised repolarization reserve capacity even if their QT_c interval is close to normal. This must be born in mind when designing pharmacotherapy for diabetic patients.

It is concluded that type 1 diabetes mellitus, although only moderately, lengthens ventricular repolarization. Diabetes attenuates the repolarization reserve by decreasing the density of I_{Ks} current, and thereby may enhance the risk of sudden cardiac death.

7. SUMMARY

According to the theory of "*repolarization reserve*" there are multiple different potassium currents, which implement a redundant mechanism to accomplish repolarization process in the normal heart. Therefore, in a healthy heart pharmacological block of a potassium current does not result in dangerous QT_c prolongation. Thus, this mechanism provides a strong safety reserve for repolarization. However, in pathological conditions where the density of one or more type of potassium current is attenuated by congenital channelopathies, remodeling or by any other heart disease, inhibition or impairment of another potassium current may cause excessive lengthening of the action potential duration and the QT_c interval leading to harmful cardiac arrhythmias.

The goals of this study were: 1) to investigate the effects of the widely used non-steroid anti-inflammatory drug diclofenac in different dog ventricular preparations on the ventricular action potential, the four main potassium currents (I_{to}, I_{K1}, I_{Kr} and I_{Ks}) and on the L-type calcium current (I_{CaL}); 2) to analyze the contribution of I_{to} to the repolarization and to the repolarization reserve in dog ventricular myocardium and finally, 3) to clarify the electrophysiological changes induced by experimental (alloxan-induced) type 1 diabetes in the rabbit.

The main results of this study show that in the normal heart, NSAID drug diclofenac does not exert marked cardiac electrophysiological effects and does not enhance risk of arrhythmia, however, in hearts where repolarization reserve is impaired, its moderate inhibition of I_{Ks} and I_{Kr} may lead to prolongation of ventricular repolarization and may also increase proarrhythmic risk.

Another message of the study is that I_{to} is involved in governing repolarization and, as a consequence, it contributes significantly to the repolarization reserve. Accordingly, inhibition of I_{to} in the presence of impaired repolarization reserve may elicit excessive repolarization lengthening resulting in EAD formation with the concomitantly enhanced proarrhythmic risk.

Finally, it is found that experimentally induced type 1 diabetes mellitus caused only a moderate but statistically significant lengthening of the QT_c interval in the rabbit heart, which was associated with a marked reduction in the density of I_{Ks}. No change in other ion currents (I_{Kr}, I_{to}, I_{CaL} and I_{K1}) was observed.

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9. ANNEX

Publications related to the subject of the Thesis