

**The importance of repolarization reserve in mammalian
ventricle**

PhD Thesis

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LIST OF PUBLICATIONS

Full length papers related to the subject of the thesis

- I. **Kristóf A**, Husti Z, Koncz I, Kohajda Z, Szél T, Juhász V, Biliczki P, Jost N, Baczkó I, Papp JGy, Varró A, Virág L. Diclofenac prolongs repolarization in ventricular muscle with impaired repolarization reserve.
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- III. N Jost, Zs. Kohajda, **A. Kristóf**, P. P. Kovács, Z. Husti, V. Juhász, L. Kiss, A. Varró, L. Virág, I. Baczkó. Atrial remodelling and novel pharmacological strategies for antiarrhythmic therapy in atrial fibrillation (review).
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- IV. Corici C, Kohajda Z, **Kristóf A**, Horvath A, Virág L, Szél T, Nagy N, Szakonyi Zs, Fülöp F, Muntean DM, Varró A, Jost N. L-364,373 (R-L3) enantiomers have opposite modulating effects on I-Ks in mammalian ventricular myocytes
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Abstracts

- I. Kristóf A, Virág L, Kovács PP, Lengyel Cs, Horváth Z, Papp JGy, Varró A
A tranzien 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)
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- III. Kristóf A, Kohajda Zs, Szél T, Husti Z, Baczkó I, Varró A, Jost N, Virág L
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INTRODUCTION

Repolarization reserve

It has been well known since the SWORD (Survival With Oral d-Sotalol) study 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or sudden cardiac death induced by non-cardiac drugs is low, several of them, such as cisapride and terfenadine were withdrawn from the market.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 arrhythmias.

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 . 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.

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.

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

- Rapid component of the delayed rectifier potassium current (I_{Kr}).
- Slow component of the delayed rectifier potassium current (I_{Ks}).
- Inward rectifier potassium current (I_{K1}).
- Calcium independent transient outward current (I_{to}).

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 I_{to} plays important role only in the early repolarization phase of the action potential because the current inactivates relatively early during the plateau. Mentioned earlier, that the current can modulate the plateau potential, and consequently may affects other important transmembrane ion currents influencing

indirectly the ventricular repolarization. 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.

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. The most consistent findings are the downregulation of transient outward and the slow delayed rectifier potassium currents. The inward rectifier potassium current is also found to be reduced in some studies.

Diabetes mellitus

Diabetes mellitus, both type 1 and type 2, is related to increased risk of sudden cardiac death, 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, which might be in the background of the increased cardiac mortality in diabetes. Lengyel et al. observed reduction of the density of transient outward and slow delayed rectifier potassium currents in diabetic dogs. 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. 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. 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, which slows down the inactivation of sodium current increasing the late sodium current (I_{NaL}).

Furthermore it is well known that lot of antiarrhythmic 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.

Top athletes

In competitive athletes, the hard training activities result in a reversible cardiac hypertrophy called athlete's heart. Cardiac hypertrophy, 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. 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. 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.

MAJOR SPECIFIC EXPERIMENTAL GOALS

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

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

RESULTS

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

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. The maximum upstroke velocity was also decreased by the drug. 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.

In dog Purkinje fibers, however, the drug significantly shortened the action potential duration and decreased V_{max} at basic cycle length of 500 ms indicating a sodium channel blocking property of the drug. The shortening of APD_{90} was rate-independent. Repolarization reserve was greatly attenuated by the application of 30 μM BaCl_2 , which partially blocks I_{K1} in dog right ventricle. BaCl_2 lengthened APD in a reverse rate-dependent manner. 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 BaCl₂. Under these circumstances the drug produced reverse rate-dependent APD prolongation.

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. Diclofenac (even at 50 μ M concentration) did not influence I_{to} or I_{K1} currents.

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}). 30 μ M diclofenac indicate a significant blockade of I_{Kr} and of I_{Ks} (at 20 mV test potential).

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. Diclofenac (30 μ M) slightly but statistically significantly decreased the amplitude of the current (at 0 mV test potential).

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

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. 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. 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. Closer inspection of the steady-state activation and inactivation relations obtained for I_{to} shows an overlap between the two curves revealing 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.

Contribution of I_{to} to repolarization reserve

Consequences of I_{to} blockade on action potential configuration were studied in canine right ventricular subepicardial muscle preparation by applying 100 μ M chromanol 293B in the presence of 0.5 μ M HMR 1556 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. Full inhibition of I_{Ks} caused only a slight lengthening of APD. Additional suppression of I_{to} by administration of 100 μ M 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.

Repolarization reserve was greatly attenuated by application of 0.1 μ M dofetilide for I_{Ks} blockade. Dofetilide markedly lengthened the repolarization of right ventricular muscles by primarily delaying phases 2 and 3, without altering the notch. This lengthening of repolarization - as expected - was reversely rate-dependent. After 40 min additional exposure to 100 μ M 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. In addition, in 5 out of 7 experiments early afterdepolarizations (EADs) were observed at cycle lengths longer than 2 s.

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

Effect of diabetes on ventricular repolarization

After 3 weeks of alloxan treatment the QT interval of diabetic animals was slightly, but significantly longer compared to that measured before alloxan administration. Since the RR intervals were also increased in the diabetic rabbits, the diabetes-induced lengthening of the QT_c interval was less pronounced, however, it was statistically significant. No significant change was observed in the control animals during the identical period of 3 weeks.

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.

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.

The amplitude of I_{to} did not differ significantly in ventricular myocytes isolated from control and diabetic rabbits (at 50 mV). The kinetics of recovery from inactivation was also not altered by diabetes.

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 μ M chromanol 293B in order to eliminate I_{Ks} , the amplitude of I_{Kr} (at 30 mV) was not altered in the alloxan-induced diabetes.

Activation kinetics of I_{Kr} measured using the envelope tail test protocol, was not altered by diabetes. 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.

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 μ M E-4031 in order to eliminate I_{Kr} . The amplitude of I_{Ks} was significantly less in diabetic than in non-diabetic rabbits at 50 mV. Activation kinetics of I_{Ks} measured using the envelope tail test protocol was not altered by diabetes. 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.

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. The density of I_{CaL} was not significantly different in the control and diabetic groups (at 5 mV).

DISCUSSION

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.

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. 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} . 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.

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.

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.

Lengthening of repolarization induced by 30 μ M chromanol 293B in the presence of I_{Kr} blockade has been previously reported in dog ventricular muscle 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} .

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.

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.

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.

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