SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Reverse rate dependency, as an intrinsic property of cardiac cells

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INTRODUCTION

Based on our present knowledge, the action potential of myocardium is formed by several ionic currents. Congenital or acquired defects of these ionic currents cause electrical signal formation and/or conduction disturbances and arrhythmias, which deteriorate the pump function of the heart and finally it can lead to death. One type of this pathological signal formation is early afterdepolarization (EAD), which is formed as the consequence of prolongation of repolarization and associated with long QT syndrome, torsade de pointes type ventricular tachycardia, and in case of progression, ventricular fibrillation. Arrhythmias formed by delayed afterdepolarization (DAD) are also common and they are the consequence of calcium overload and most frequently they occur after myocardial ischaemia/reperfusion. The delayed afterdepolarizations can activate easily reentry type signal conduction disturbances. The aboved mentioned life-threatening conditions are ceased or prevented by mainly drugs having effect on ion channels of cardiac myocytes. These antiarrhythmic agents are classified according to Vaughan Williams into four classes based on their effect mechanism.

Since the SWORD (Survival With ORal D-sotalol) trial it is generally known that class 3 antiarrhythmic agents exhibit reverse rate dependent lengthening of the action potential duration (APD) and refractery period. This indicates that changes in APD are greater at slower than at faster heart rates. Therefore in case of tachycardia when cycle lengths are pathological short and the antiarrhythmic effect is most desirable, these agents have no proper effect on the lengthening of the repolarization and effective refractery period. However, in case of bradycardia when cycle lengths are long, the extremly prolonged repolarization can lead to formation of early afterdepolarization and life-threatening ventricular arrhythmias (tachycardia). Since the proarrhythmic potential of class 3 antiarrhythmic agents is probably related to their reverse rate dependent (RRD) lengthening of action potential duration, the development of a class 3 agent without reverse rate dependent effect would be desirable. However, the exact electrophysiological mechanism responsible for reverse rate dependency of class 3 antiarrhythmic drugs has not been

clarified. For that reason, the detailed description of the underlying mechanism is really important, because probably it should provide a chance to develop more effective antiarrhythmic drugs.

LITERARY REVIEW

The rate dependency of the antiarrhythmic drug-ion channel interaction in the modulated receptor theory and in the guarded receptor theory

The modulated receptor theory states that antiarrhythmic drug affinity for a specific receptor on the channel protein is modulated by the channel state. The basic idea behind the guarded receptor theory is access to a channel binding site is controlled by the channel conformation. Drug access to the channel and its binding and unbinding rates depends on the state of the channel, which is dynamically changing during the cardiac cycle. If the channel block developed preferentially during the diastole and dissipated during the action potential, reverse rate dependent prolongation of APD would be expected. Furthermore, during repetitive stimulation, the fraction of ligand-bound channels follows an exponential time course, determined by the interstimulus interval, channel-gating processes, drug concentration, and the forward and reverse rate coefficients, which are characteristic in the binding process.

The modulated receptor theory opposed by the recognition that many drugs with different chemical structure display reverse rate dependency. However, it is not likely that the binding of all agents occur in the same channel state. Nevertheless, this hypothesis is not negligible because from many Na^+ and Ca^{2+} blockers are known to display actions of rate dependent nature.

I_{Ks} acummulates at fast heart rates

In 1993, Jurkiewicz and Sanguinetti have presented data that class 3 antiarrhythmic agents containing a methanesulfonamidophenyl structure exhibit reverse rate dependent lengthening of action potential duration of

cardiomyocytes isolated from guinea pigs. Therefore the effects of antiarrythmic agents were more pronounced on low stimulatory frequency than on a higher frequency. In these experiments the authors applied dofetilide, which is a selective blocker of the rapid delayed rectifier K^+ current in the used concentration (0.1 μM) and has no effect on I_{Ks}. Voltage-clamp experiments supported the view that dofetilide blocks I_{Kr} selectively (IC_{50}=31.5 nM), but it has no significant effect on I_{Ks} and I_{K1}. Applying different stimulatory frequency they found that neither the amplitude of I_{Kr}, nor the amplitude of I_{K1} did not change, moreover the I_{Kr} inhibiting effect of dofetilide was independent of the magnitude of the stimulation frequency. In contrast, the amplitude of I_{Ks} increased by the elevation of stimulatory frequency. This finding was explained by the incomplete deactivation of I_{Ks}. Based on the experimental results they concluded that inhibition of the I_{Kr} is responsible for the prolongation of action potential when dofetilide was applied. Significant accumulation of I_{Ks} may occur due to the incomplete deactivation of the current at fast heart rates, which would greatly attenuate the APD lengthening effect of I_{Kr} blockade. Therefore I_{Ks} plays a relatively bigger role in the process of repolarization at high stimulatory frequency, in this way the action potential lengthening effects of I_{Kr} inhibitors can decrease. This theory is strongly opposed by the observation that reverse rate dependency was also evident after the complete suppression of $I_{Ks.}$

K^+ accumulates in the extracellular space at high stimulatory frequency

In 1996, Yang and Roden studied the effects of a nonspecific inhibitor of I_{Kr}, quinidine, and a specific I_{Kr} blocker, dofetilide, on AT-1 cells expressing hERG channels, where contaminating outward currents are absent. The authors concluded that increase of extracellular potassium shifts the dose-response curve of both agents to right. Elevating K^+ in the extracellular space from 1 to 8 mmol/L increased the IC_{50} for dofetilide block from 2.7±0.9 to 79±32 nmol/L and for quinidine block from 0.4±0.1 to 3.8±1.2 μmol/L. At higher heart rates

when homeostatic mechanisms such as sodium-potassium pumpe, have not yet maximally compensated, K^+ can accumulate in the extracellular space (as it can be observed in ischaemia, when K^+ flows out from damaged cells). Elevation of potassium occurs during myocardial ischemia or at rapid heart rates, cause a decrease in the efficiency of I_{Kr} blockers. In case of hypokalaemia low potassium concentration increases the sensitivity to I_{Kr} blockers which can help to explain a well-known clinical phenomenon that hypokalaemia and bradycardia increase the risk for genesis of bradycardia-dependent arrhythmias such as torsade de pointes. The authors give two possible explanations for the mechanisms whereby changes in extracellular potassium modulate the reverse use dependent block of $I_{Kr.}$ One possibility is that a potassium-induced change in the conformation of the channels responsible for I_{Kr} could alter the access of the blocking drug to its target site on the channel. The other possible explanation could be that these drugs could bind to open channels in a voltage dependent manner. In this case the increasing potassium, permeating through the channel, might impair the binding of a blocking drug to a site in the pore. This theory is opposed by the findings that not only I_{Kr} blockers exhibit reverse rate dependency.

Rate dependency of kinetic parameters of potassium currents

Rocchetti et al, (2001) have investigated rate dependency of delayed rectifier currents during the guinea pig ventricular action potential using action potential clamp technique. They have presented data about earlier activation of I_{Kr} and I_{Ks} during action potential and both currents amplitude increase when cycle length is shortened (high heart frequency), however, the relative contribution of I_{Kr} and I_{Ks} to repolarization does not change. The mechanisms by which I_{Kr} and I_{Ks} increase at shorter cycle lengths are profoundly different. Decreasing of diastolic interval with the same action potential duration, I_{Kr} remained unchanged but I_{Ks} was further increased, so I_{Ks} amplitude depends on the ratio between systolic and diastolic times. In contrast, shortening of the action potential duration by keeping the diastolic interval constant increased the I_{Kr} amplitude. Increase of I_{Ks} might be due to the accumulation of activated

channels when shortening of the diastolic interval. The authors also mentioned that this explanation may not apply to other species like canine or human cardiomyocytes in which I_{Ks} deactivation kinetics are considerably faster than in the guinea pig. However, it should be recognized that *in vivo*, additional factors are likely to contribute to the enhancement of I_{Ks} at faster rates. These may include I_{Ks} sensitivity to increased intracellular Ca^{2+} concentration and β-adrenergic activation. The I_{Kr} has special gating properties (as ultrafast inactivation), so during the plateau phase of repolarization (when repolarization is slow < 1 V s^{-1}) the magnitude of I_{Kr} decreases and decelerates the process of repolarization. However when repolarization became very fast (> 1.5 V s^{-1}), it causes significant increase of I_{Kr}. A positive feedback may exist between I_{Kr} and the magnitude of repolarization, causing the effects of I_{Kr} inhibition to be autoregenerative. However, the magnitude and kinetic parameters of I_{Kr} widely differ in species.

Virág et al. (2009) have found that action potential configuration may influence the reverse rate dependent APD prolongation due to the intrinsic properties of I_{Kr} and I_{K1} currents. Drugs lengthening repolarization by decreasing repolarizing outward, or increasing depolarizing inward currents are expected to cause reverse rate dependent APD lengthening with high probability, regardless of which current are modified.

I_{K1} has special kinetic properties similar to I_{Kr} as Rocchetti and her colleagues described. In the case of I_{Kr}, this was attributed to the faster time course of inactivation and recovery from inactivation than that of activation and deactivation. In the case of I_{K1}, the rapid binding of magnesium ions and polyamines to the channel was proposed to occur at less negative membrane potentials, which very rapidly occludes, and thereby inactivates the channel. Switching to positive potentials, I_{Kr} slowly activates but the channels rapidly inactivate. When repolarization proceeds with steeper slope, a larger amount of recovery from inactivation can occur per unit time than in the case of a less steeper slope. Consequently, this results in more outward current at steep than at less steep repolarization, resulting in further acceleration of repolarization. Deactivation of the channels upon repolarization is a much slower process and therefore it may influence the developing current only to a

limited extent. In the case of the I_{K1} channels, the fast block, induced by magnesium ions and polyamines at positive voltages, becomes relieved quickly upon by the increase of the slope of repolarization resulting in more I_{K1} at steeper repolarization rates. Therefore, any factor decreasing the slope of repolarization, which can be independent of K^+ channel block, such as the enhancement of the late sodium current after application of veratrine or occurring in patients during heart failure and long QT 3 syndrome would further diminish the repolarization force. It is important and the authors also emphasize that the above described behaviour of I_{Kr} and I_{K1} may not be the only cause of the reverse rate dependent repolarization lengthening, especially at higher frequencies. However, it may contribute to the RRD as an amplifier by magnifying and accelerating the consequences of a small drug-induced imbalance of inward and outward currents during the plateau phase.

AIM

In spite of the several theories developed so far to explain the frequency dependent nature of drug-effects on APD, the underlying mechanism has not yet been clarified. The aim of the present work was to study all theories regarding the rate dependent properties of APD modulation and to elaborate a hypothesis which can interpret the previously published experimental results. Properties of ventricular myocardium of several mammalian species, including human, dog, rabbit, guinea pig and rat were compared. Although we know that electrophysiological properties of canine cardiac preparations are believed to most resemble those of the human ones, experiments were carried out on several species to study how can the difference between action potentials of different species (absence or existence of a particular ionic current) influence the modulation of action potential duration.

During experiments we had opportunity to answer the following specific questions: is the frequency dependent nature of drug-effects of APD a direct consequence of changing the stimulatory frequency, or is related to the concomitant shift in baseline APD? Is the phenomenon evident only under steady-state conditions or it can also be observed following abrupt changes of the cycle length?

These questions can be answered by analyzing the rate dependency of APD modulation by drugs that either enhance or block a variety of cardiac ion currents and by transmembrane current injection. We also studied the relationship between APD and net membrane current flowing during action potential plateu phase.

METHODS

Animal tissue preparations

Experiments were carried out on adult, purpose-bred dogs (10-15 kg), guinea pigs (0.3-0.5 kg), rabbits (2-3 kg), and Wistar rats (0.3-0.5 kg). After the chest had been opened, the heart was rapidly removed and placed in cold (5°C) Tyrode solution. Papillary muscles and Purkinje fibers excised from both ventricles, were used for standard microelectrode measurements. A wedge-shaped section of the left ventricular wall of canine hearts, supplied by the left anterior descending (LAD) coronary artery, was dissected and cannulated for isolation of single myocytes. The canulated left anterior descending coronary artery was perfused with Ca^{2+}-free JMM solution (Minimum Essential Medium Eagle; Joklik modification) during the initial 5 min of perfusion to remove Ca^{2+} and blood from the tissue. Cell dispersion was performed for 30 min in the same solution containing, in addition, collagenase. During the isolation procedure, the solutions were gassed with carbogen and the temperature was maintained at 37 °C. Until use cells were stored in Minimum Essential Medium solution at 14 °C.

The human experimental samples were obtained from general, undisesed organ donors, their valves were utilized for pulmonary and aortic valve transplantation surgery. Before explantation of the hearts the patients did not receive any medication except for dobutamine, furosemide and plasma expanders.

Electrophysiological measurements

Cardiomyocytes were sedimented in a plexiglass chamber, allowing continuous 10 ml/min superfusion with oxygenized Tyrode solution at 37 °C. Intracellular borosilicate glass microelectrodes, filled with 3 M KCl and having tip resistances of 20-40 MΩ were used to record transmembrane potentials. During examining frequency dependency the cycle length (CL) was set to 5 s, than it was gradually reduced until reaching 0.3 s. Before starting the measurements, at least 100 cycle were waited to allow the cells to equilibrate. The effects of current pulses on action potential were also examined. These

current pulses (having amplitudes of either -40 or +100 pA, respectively) were injected from the upstroke of every 20th action potential. Thus, action potentials with current injections were compared to 19 regular action potential waveforms.

Papillary muscles and Purkinje strands were mounted in a plexiglass chamber, allowing continuous superfusion with oxygenized Tyrode solution at 37 °C. The preparations were paced through a pair of platinum electrodes. The pacing CL was between 5 and 0.3 s. In case of Purkinje strands the longest CL was 3 s. Glass microelectrodes, filled with 3 M KCl and having tip resistances of 5–20 MΩ were used to record transmembrane potentials. Action potential duration at 90 % and 50 % of repolarization (APD_{90} and APD_{50}) were determined. All drugs on isolated cardiomyocytes were applied at least for 2-3 min, until the complete effect developed. In case of multicellular preparations after taking control records the preparations were superfused for 30-40 min with the drug tested and the measurement was repeated.

Numerical stimulations

The purpose of the numerical simulations was to test the working hypothesis that such a general mechanism could be provided by the features of the relation linking net transmembrane (I_{net}) current to APD. 'Classical' action potential models were not suitable for this purpose because they include a description of specific channel gating properties potentially contributing to RRD. Exclusion of such a contribution was strictly required for unequivocal testing of the working hypothesis, which is by definition independent of specific channel gating mechanism. The used approach was a calculation of the point-by-point change in the time-course of repolarization caused by addition of a constant current. The procedure was applied to real action potential waveforms, previously acquired from canine myocytes at two CLs. The simulation is based on the following principles. In a single cell (i.e. under space-clamp conditions), total membrane current during the action potential is zero, therefore the net transmembrane ionic current (I_{net}) equals with the capacitive current (I_c) in an opposite sign. The capacitive current can be calculated by multiplying membrane potential velocity by membrane electrical capacitance ($I_{net} = -C_m * dV/dt$). This implies that, in a single myocyte

(with constant C_m), membrane potential velocity ($V_{m'}$) is representative of net transmembrane current (I_{net}), therefore the latter can be estimated by differentiation of the action potential waveform. During repolarization membrane capacitance is charged by I_{net}; since this process is continuous in time I_{net} magnitude sets the time required for achievement of a given V_m difference. A digitized action potential waveform is a $V_m(t)$ matrix in which t is finite and determined by the sampling interval (1 ms). The simulation was implemented as follows (the term 'array' is used for a matrix of two dimensions e.g. V_m and t). The term 'vector' refers to a mono-dimensional matrix; positions within a vector are defined by the index 'i' the repolarization phase of endocardial action potentials recorded at CLs of 3 and 5 s from the same impalement (sampling rate 1 kHz) were used as control waveform arrays. The effect of adding a constant inward I_{net} was calculated as a delay in the achievement of each V_m value during repolarization; the calculation was repeated for all the values of V_m to obtain a time vector defining the repolarization courses during injection of the test I_{net}: $t_{stim}(i) = t_{cont}(i-1) + \Delta V_m / V_{m'(i)}$ where t_{cont} is the control time vector and dV_m is the V_m change between two consecutive time points. The repolarization course during current injection is thus described by $V_m(t_{test})$ arrays, one for each CL, compared with the respective $V_m(t_{cont})$ arrays. The cumulative point-by-point change in repolarization time caused by current injection was calculated by digital subtraction of the respective time vectors ($t_{test(i)}$-$t_{cont(i)}$). Differentiation of the subtraction vector provides an estimate of how the impact of a change in I_{net} on APD may vary as a function of the portion of repolarization during which it occurs.

Statistical analysis

All values presented are arithmetic means ± standard error of the mean. Statistical significance of differences was evaluated by using one way analysis of variance followed by Student's t-test for paired or unpaired data, as appropriate. Linear regression was used to determine the correlations between data. Differences were considered significant when the P value was less than 0.05.

RESULTS

Not only class 3 antiarrhythmic drugs show a reverse rate dependent lengthening of action potential duration

As it was described in the introduction part, class 3 antiarrhythmic agents are known to lengthen of action potential duration more pronounced at lower heart frequency, than at higher frequency. Therefore the examined class 3 antiarrhythmic agents exhibit reverse rate dependent lengthening of the action potential duration. However the phenomenon of reverse rate dependency is not restricted to I_{Kr} blockade, but is observed with several drugs that lengthen APD independently of the underlying ionic mechanism.

Since the canine ventricular myocardium resembles most the human one regarding its electrophysiological properties, the frequency dependent effects of various APD lengthening agents are demonstrated in canine ventricular papillary preparations first. Our experiments demonstrate that the window I_{Na} activator veratrine (1µg/ml), the I_{Ca} activator Bay K 8644 (1µM), the I_{Kr} blocker dofetilide (1µM) and I_{K1} blocker $BaCl_2$ (10 µM) display clearly reverse rate dependent action on APD, i.e. the lengthening of APD increased monotonically with increasing the cycle length of stimulation. These results clearly indicate that the reverse rate dependent lengthening of APD is not restricted to I_{Kr} blockade, but is observed with any drug that is able to lengthen APD.

Not only APD lengthening agents show reverse rate dependent properties

During our experiments we tested that whether only the frequency-dependent lengthening of APD or also its shortening shows the properties of reverse rate dependence (RRD). To answer this question APD shortening effects were examined on several species. Lidocaine is known to suppress the window Na^+ current, while nicorandil is a frequently used ATP sensitive K^+ channel ($I_{K(ATP)}$) opener. The APD shortening effects of these agents showed reverse rate dependency similar to APD lengthening drugs in canine Purkinje fiber. At short cycle length (0.3 s) the APD shortening effect of lidocaine was at

proximately 30% of values measured at longer cycle length (3 s). Nicorandil showed the same result. Lemakalim having a similar mechanism of action to nicorandil also showed more pronounced shortening at 3 s CL than at 0.3 s CL in guinea pig preparation. In human ventricular preparations the same results were found, the shortening effects of the I_{Na} blocker mexiletine and tetrodotoxin on APD were reversely rate dependent. Therefore we can conclude that not only the lengthening, but also the drug induced shortening of APD is reversely rate dependent.

APD modulation by current injection

According to results desribed above, the APD changes were more pronounced at longer pacing cycle length than at shorter cycle length. This conclusion was based on experiments in which APD was modulated by pharmacological agents. Previous interpretations of RRD were based on the features of drug-channel interactions. To test this hypothesis inward and outward current pulses were used to lengthen or shorten APD, respectively.

Inward (-30 pA) and outward (+60 pA) current pulses were injected throughout repolarization to lengthen and shorten APD, respectively. The cycle length was initially set to 5 s, and it was continuously varied to the shorter values until 0.3 s. The effect of current injection on APD was reversely rate dependent. Results of experiments indicate that the lengthening or shortening effect of a given inward or outward current pulse is increasing with increasing the pacing CL, when the action potentials are sui generis longer. This finding suggests that the magnitude of APD prolonging effect is exclusively a function of the initial APD and it is independent of the nature of the current change (electrical impulse or drug action).

APD prolongation by $BaCl_2$ is the consequence of I_{K1} blockade. Neither I_{K1}, nor its inhibition by $BaCl_2$ are significantly rate dependent; thus making this experiment best suited to unveil a mechanism of RRD independent of specific channel or drug properties. In the absence of current injection, $BaCl_2$ prolonged APD and made its dependency on CL steeper i.e. $BaCl_2$ effect showed clear cut RRD. The $BaCl_2$-induced prolongation of APD was

completely offset at all CLs by concomitant injection of the same amount of current (39±3 pA).

At the same time, current injection also reduced the steepness of its CL dependence back to control value, thus eliminating RRD of the $BaCl_2$ effect. This implies that the current change induced by $BaCl_2$ was the same at all CLs; thus, the resulting APD prolongation was only a function of control APD value, which in turn proportional to CL.

Reverse rate dependency is not restricted only to one species

As described above, the I_{Kr} blocker dofetilide, the I_{K1} blocker $BaCl_2$, the I_{Na} activator veratrine, or the I_{Ca} activator Bay K 8644 shared a common feature: their lengthening effects on APD showed reverse rate dependency in canine papillary ventricular muscle. Increasing of CL the APD modification increased monotonically. A similar effect was found in canine Purkinje fiber, $I_{K(ATP)}$ activator nicorandil and the dominantly I_{Na} blocker lidocaine also showed rate dependency and this effect was also cycle length dependent. In human cardiac multicellular preparation both drug-induced lengthening by I_{Kr} blocker dofetilide (50 nM), d-sotalol (30 µM), E-4031 (1µM), I_{K1} blocker $BaCl_2$ (10 µM) and shortening by I_{Na} blocker mexiletine (10 µM), and tetrodotoxin (TTX) (2 µM) of action potentials displayed RRD, i.e. changes in APD were greater at longer than at shorter CL. In guinea pig multicellular ventricular preparations d-sotalol lengthened the APD at all applied CL and the $I_{K(ATP)}$ activator lemakalim shortened the APD at all pacing rate. The magnitudes of changes were greater at longer CL in case of both agents. We can conclude that the effect of the applied agents showed reverse rate dependency in guinea pig ventricular papillary muscle in spite of the fact, that the set of ion currents underlying the action potential in guinea pig cardiomyocytes is markedly different from that found in dog and human. In guinea pig both the type of expressed channel proteins and the kinetic properties and density of ion channels are different from those found in the aboved discribed species. The finding of these experiments indicates that RRD is a general property of APD modulation and independent of species.

Reverse rate dependency is not characteristic of all species

RRD of action potential changing in rabbit myocardium is markedly different from those observed in guinea pig, dog and human. APD displayed a non-monotonic dependency on CL in rabbits, maximum APD being achieved at an intermediate CL between 0.5 and 0.7 s. This provided a unique opportunity to test whether rate dependency of drug effects is directly determined by pacing rate, or RRD is an indirect consequence of pacing frequency. D-sotalol and lemakalim were used to prolong and shorten APD, respectively. As expected, d-sotalol (20 μM) lengthened and lemakalim (15 μM) shortened APD in papillary muscles of rabbit at all pacing rate. In rabbit preparations the maximal drug-induced APD changes occurred at an intermediate CL between 0.5 and 0.7 s, at which the baseline APD was the longest. Important to note that drug-induced APD_{90} changes also displayed reverse rate dependency similarly to control. When APD_{90} changes were plotted against control, the greatest value was measured not at the longest CL, but between 0.5 and 0.7 s. Therefore the drug-induced maximal APD change is achieved at an intermediate CL, when baseline (pre-drug) APD was the longest in rabbit papillary muscle. Lemakalim (15 μM) was shown to shorten and d-sotalol (30 μM) to lengthen APD in rabbit papillary muscles in the greatest extent at the longest baseline APD (around 195 ms), while drug-induced APD change is decreasing with shorter baseline APD values. These findings suggest that the magnitude of drug-effect depends on baseline APD, rather than on pacing rate.

The magnitude of APD modification depends on the baseline APD

According to previous experimental results that drug-induced change of APD seems to be strictly determined by the pre-drug APD value rather than by CL – as it has been initially proposed. When APD changes by veratrine, Bay K 8644, dofetilide and $BaCl_2$ were plotted against baseline APD in canine papillary myocardium a similar correlations were found to those observed in rabbit. In human multicellular ventricular preparation, plotting the APD lengthening or shortening effect of dofetilide, d-sotalol, E-4031, $BaCl_2$, TTX and mexiletine as a function of the respective control (pre-drug) APD indicated

that lengthening and shortening of APD was always proportional to the control APD measured before drug treatment. Similar results were obtained from guinea pig multicellular ventricular preparations.

In contrast, when APD changes were studied as a function of baseline APD in guinea pig, both agents were linearly proportional to baseline APD. Therefore APD changes were largely proportional to the pre-drug APD in case of guinea pig, too.

In the previously described experiments the changes in APD induced by the inward (-30 pA) and outward (+60 pA) current pulses and baseline APD_{90} showed positive correlation, the relation between the two parameters was linear in the range of studied frequencies. Present results suggest that the magnitude of APD modification is exclusively a function of the baseline APD, and independent of the nature of current change (electrical impulse or drug action), the gating kinetics and profiles of the involved ion current, or properties of the applied drug.

Rate dependency of rat ventricular myocardium

Rat ventricular muscle was chosen because it has a set of ion currents markedly different from those of other species, and its APD is shorter by one order of magnitude than that of the “plateau-forming” larger mammals. In the background of this phenomenon stands the fact that rat myocardium express different ion channels and different intracellular calcium homeostasis. Moreover, and most importantly, APD increases at higher heart rates in rats opposite to many other species. To test the aboved mentioned differences we examined the rate dependency of rat ventricular muscle. Furthermore, restitution of APD was studied, in contrast to previous experiments where preparations were stimulated by constant cycle length. In this experiment the preparations were continuously paced using a train of 20 basic stimuli delivered at a frequency of 1 Hz. These trains of basic stimuli were interrupted by a single extra stimulus applied at successively longer coupling intervals. In this way, each 20th basic action potential was followed by a single extra action potential occurring at successively longer diastolic intervals (DI). DI was defined as a time elapsed from repolarization of the last basic action potential

of the train to the upstroke of the extra action potential. Restitution curves were generated by plotting the duration of each extra action potential against the respective DI.

When DI was increased from 50 ms to 4 s APD monotonically decreased in rat ventricular muscle preparations. APD was increased by tetraethylammonium and 4-aminopyridine, both superfused for 60 min. APD was shortened by nifedipine and $MnCl_2$ at each DI. The magnitude of the drug-induced lengthening and shortening of APD was greater at shorter than at longer DI values, when the control APD measured before drug treatment was longer. The APD modifying effects of all drugs showed positive rate dependent properties in rat (they were more pronounced at shorter DI values than at longer ones), while their magnitudes were still proportional to the duration of the pre-drug action potential.

Relationship between the net membrane current and APD

Equations $I_{net} = -C_m * dV/dt$ and $APD = -\Delta V_m * C_m / I_{net}$ predict that APD depends on net membrane current (I_{net}). This prediction was tested by plotting I_{net} at 50% of APD ([I_{net50}, calculated from V_m) as a function of APD at various CLs and in the presence or absence of inward and outward current pulses or inhibitors. I_{net50} was inversely related to APD according to a non-linear relation, and I_{net50} values fell on the same curve irrespective of whether APD changes were induced by pacing, by current injection (either outward or inward) or by ion channel blocker or activator. The APD changes can be examined by studying the effects of the above described channel inhibitors on human papillary muscle and on canine ventricular myocardium. The I_{net}-APD relationship was tested by pharmacological treatment, by injection of current pulses (-30 and +60 pA), and by changing of pacing CL (between 0.4 and 5 s). The shape of the I_{net}-APD relationship was preserved during all experimental conditions, which indicates that the I_{net} value measured at APD_{90} is independent of experimental conditions. The value of I_{net} depends exclusively on APD.

Numerical simulations

The purpose of numerical simulations was to examine the relation between I_{net} and APD. When the same inward current (-0.066 pA/pF) was added to I_{net}, APD_{90} was prolonged more at a CL of 5 s (35.4 ms) than at a CL of 0.3 s (17.4 ms), which demonstrates the reverse rate dependency of APD changing. Similar results were obtained in another series of experiments, RRD of APD changes was smaller for outward current injection (to shorten APD) when compared with inward current injection (to lengthen APD).

Injection of -30 pA current caused the same APD prolongation as APD shortening induced by injection of +60 pA current. Thus, the model also predicts asymmetry of RRD changes between inward and outward current injection.

Most ion channel currents are time-dependent, thus, they have a specific time profile during repolarization. The mechanism of RRD predicts that such profile may be important in determining the APD response to current modulation. The analysis predicts that APD is most sensitive to I_m changes affecting phases with slower repolarization. Moreover, it shows that because of its non-monotonic course, the spike-and-dome phase of repolarization is very sensitive to I_m changes. If restricted to this phase, an inward change in I_m may paradoxically shorten APD. Such negative portion was larger at the longer CL, because of the deeper notch between the spike and the dome; however, the time profile of the spike-and-dome phase may also change independently of CL (e.g. considering of hetereogeneity of ventricular wall), therefore, generalization of this feature as a property of intrinsic RRD may be unwarranted.

CONCLUSION

The multitude of theories developed so far to explain reverse rate dependency indicates that its exact mechanism remained to be elucidated. Previous interpretations of RRD were based on the features of the blocked channel or drug-channel interaction. A general difficulty with such interpretations was that drug-channel interaction is, in most of the cases, directly rate dependent and therefore unsuitable to account for RRD. For instance, a major disparity in the RRD of APD prolongation by I_{Kr} and I_{Ks} blockers is a feature of guinea pig myocytes and was initially attributed to the different rate dependency of the two currents. Whereas these experiments used a conventional voltage clamp approach, later ones performed by action-potential clamp showed that guinea pig I_{Kr} and I_{Ks} conductances are similarly increased at faster rates and that the proportion of total current provided by each of them is rate independent. According to these observations, rate dependency of I_{Kr} and I_{Ks} may be unsuitable to explain RRD of APD modulation. In canine ventricular myocytes, I_{Kr} and I_{K1} provide most of repolarizing currents and are rate independent; nevertheless, their blockade affects APD with clear-cut RRD. Intrinsic rate dependency of persistent Na^+ current is relatively small, while lidocaine and veratrine preferentially bind to Na^+ channels at higher heart rates, however, the effects of lidocaine and veratridine on APD show remarkable RRD. To summarize, although RRD of APD modulation has been reported for many agents with different mechanisms of action, a unifying interpretation of the phenomenon is missing.

The aim of the present work was to elaborate a hypothesis which can interpret this phenomenon under all conditions. Here we address such a hypothesis by analysing the rate dependency of APD modulations by drugs that either enhance or block a variety of cardiac ion currents in myocardium of several mammalian species including human, dog, rabbit, guinea pig and rat. Moreover, we test the effects of transmembrane current injection on isolated cardiomyocytes.

Since the canine ventricular myocardium resembles most the human one regarding its electrophysiological properties, the frequency dependent effects

of various APD lengthening agents are demonstrated in canine multicellular ventricular preparations first. The I_{Kr} blocker dofetilide, I_{K1} blocker $BaCl_2$, the I_{Ca} activator Bay K 8644 and the I_{Na} activator veratrine display clearly reverse rate dependent action on APD, i.e. the lengthening of APD increased monotonically with increasing the cycle length of stimulation, independently of the drug applied or the ion channel activated or blocked. This reversely rate dependent prolongation of APD was equally observed if the change in APD was expressed in absolute value (in ms) or in a percentage form. These results clearly indicate that the reverse rate dependent lengthening of APD is not restricted to I_{Kr} blockade and to class 3 antiarrhythmic drugs, but is observed with any drug that is able to lengthen APD.

In another preparation, in free running canine Purkinje strands, drugs known to shorten the cardiac action potentials were tested. Lidocaine is known to suppress the window Na^+ current, while nicorandil is a frequently used ATP-sensitive K^+ channel opener. The effects of these agents on APD were not only similar in size, but their frequency dependence was also almost identical: both drugs shortened APD stronger at longer than at shorter cycle lengths. In other words, not only the frequency dependent lengthening of APD, but also its shortening shows the properties of reverse rate dependence. Basically similar results were obtained when a variety of APD lengthening and shortening agents were tested in other mammalian cardiac preparations including papillary muscles of the guinea pig and those derived from undiseased human hearts. All the agents applied to lengthen (the I_{Kr} blocker dofetilide, d-sotalol, and E-4031, the I_{K1} blocker $BaCl_2$) or shorten (the I_{Na} blocker mexiletine and tetrodotoxin) APD shared the property of reverse rate dependence. At this point one might conclude that the reverse rate dependent nature of drug-action in the heart is a general property of mammalian cardiac tissues, independent of the species or preparation used, the ion channel modified, or the direction of the resultant APD change. In the following rabbit myocardium was examined because APD changes display a non-monotonic dependency on cycle length in rabbits, maximum APD being achieved at an intermediate cycle length between 0.5 and 0.7 s. These findings are markedly different from those found in dog and human. This provides a unique opportunity to discriminate between

rate dependency and APD dependency of drug-induced APD changes. In this case, d-sotalol and lemakalim were used to prolong and shorten APD, respectively. As expected, d-sotalol lengthened and lemakalim shortened APD in rabbit papillary muscles at all frequencies. However, in contrast to results obtained in canine, human, and guinea pig preparations, where the APD lengthening and shortening displayed monotonic reverse rate dependency, the maximal drug-induced APD changes in rabbit preparations occurred at the intermediate cycle length between 0.5 and 0.7 s, at which the baseline APD was the longest. According to this result the magnitude of drug-effect seems to depend on the duration of the baseline action potential, rather than on the pacing rate, suggesting that longer action potentials are more sensitive to modulation than shorter ones, irrespective of the actual pacing rate. The above hypothesis can be best examined by studying the frequency dependent properties of actions of various K^+ and Ca^{2+} channel inhibitors on APD during the electrical restitution process of rat ventricular muscle. This preparation was chosen because (1) it has a set of ion currents markedly different from those of other species, and consequently, (2) its APD is shorter by one order of magnitude than that of the plateau-forming larger mammals, and most importantly, (3) its APD increases at higher heart rates - opposite to many other species. Therefore, the APD lengthening effect of the K^+ channel blocker 4-aminopyridine and tetraethylammonium, as well as the APD shortening effect of the Ca^{2+} channel blocker nifedipine and $MnCl_2$ was studied in rat as a function of the diastolic interval, a parameter indicating the proximity of the action potentials. As could be expected from the negative APD-cycle length relationship in rat, all drug-induced APD changes were largely *inversely* proportional with the diastolic interval. This result is the opposite of that we have previously seen in canine, guinea pig, and human preparations, where the drug-induced changes were roughly *directly* related to cycle length. However, when these drug-induced APD changes were studied as a function of the baseline APD in rat, the larger drug effects were observed in cases of the longer baseline action potentials – similarly to results obtained in the other mammalian species. This indicates that dependency of APD on cycle length or diastolic interval and its modulation by drugs are tightly coupled with cycle

length and diastolic interval acting as the modulator of the baseline (pre-drug) APD and the latter directly determines the magnitude of drug-induced changes. This view is reinforced by drug-induced APD changes are demonstrated to be proportional to the baseline (pre-drug) APD value in all the preparations studied so far (dog, human, guinea pig, rabbit, and rat) independently of the pacing cycle length or diastolic interval applied.

When studying the effect of an ion channel activator or blocker on action potential configuration, it can be considered as if we have added or removed an inward or outward current to/from the net membrane current flowing during the action potential plateau. This was tested in experiments performed in enzymatically isolated canine ventricular myocytes, impaled with sharp microelectrodes. In these experiments inward current pulses of -30 pA amplitude were injected into the myocytes in order to lengthen APD. Or alternatively, outward current pulses of +60 pA amplitude were injected in order to shorten action potentials. All these measurements were performed at various pacing cycle lengths. The effect of a current injection on APD was reversely rate dependent. Furthermore, the changes in APD induced by the inward and outward current pulses were linearly proportional to baseline APD values measured prior to current injection. Plots generated this way were almost indistinguishable from those obtained with an ion channel agonist or antagonist, indicating that the magnitude of APD modification is exclusively a function of the baseline APD, and independent of the nature of current change (electrical impulse or drug action), the gating kinetics and profiles of the ion current involved, or properties of the drug applied. This point is further demonstrated by the effects of reversing the $BaCl_2$-induced prolongation of APD by applying an injection of an outward current pulse. The $BaCl_2$-induced prolongation of APD was completely offset at all cycle lengths by the concomitant injection of the same amount of current. APD prolongation by $BaCl_2$ is the consequence of I_{K1} blockade. Since, neither I_{K1}, nor its inhibition by $BaCl_2$ are significantly rate dependent, the resulting APD prolongation could be only influenced by the baseline value of APD, which, in turn, was determined by the pacing cycle length. In another series of such experiments APD modifications induced by abrupt changes in the cycle length could be fully

compensated by injections of current pulses having the appropriate amplitudes.

If the magnitude of an APD change of any kind of origin is in fact proportional to the baseline value of APD, it must somehow be related to the change in the net membrane current flowing during the action potential plateau. I_{net} was determined at half duration of the action potential from the slope of the middle portion of the plateau using the following simple equation: $I_{net} = -C_m*dV/dt$, where C_m is membrane capacitance and dV/dt is the momentary rate of membrane potential change (first time derivative). When I_{net} is expressed in pA/pF, its magnitude equals to the negative slope of the membrane potential change at any point during the action potential. The assumption that APD modulation reflects a change in I_{net}, independently of the specific current affected, was verified by plotting I_{net} against baseline APD under a variety of experimental conditions: at various pacing cycle lengths, before and after applying ion channel blockers or activators and in the presence or absence of inward or outward current pulses. The APD-I_{net} relationship followed a hyperbolic function and this shape was preserved independently of the experimental conditions applied (drug effect or current injection) and preparations (human papillary muscle, canine Purkinje fiber, isolated canine cardiomyocytes). Based on the results above reverse rate dependency of drug effects is a pure consequence of the reverse rate dependent behavior of APD itself, namely because APD is greater at longer than at shorter cycle lengths in the majority of larger mammals including canine and human ventricular myocardium. Indeed, such changes were observed in rat (and in rabbit within the range of 0.7-3 s), where the applied agents showed not reverse but direct rate dependency. All these were confirmed under not only steady–state circumstances but under restitution conditions which is a better model for extrasystole. Thus the explanation for the reverse rate dependency is quite simple and mechanistic. Since I_{net} flowing during the plateau is smaller in the case of a longer than a shorter action potential, a given current added or blocked (either as a current pulse, or due to a pharmacological intervention) is expected to cause a larger relative displacement of I_{net}, and consequently, a greater change in APD in case of a

longer initial action potential. Therefore, the reverse rate dependent nature of drug-action can be considered as a general intrinsic property of canine and human cardiac cells. Since the drug-induced changes in APD well correlated with baseline APD in all of the species studied regardless of the ion current modified or the agent used, one may conclude that reverse rate dependency may simply be a consequence of the inverse relationship existing between I_{net} and APD. This confirms the prediction that APD modulation by drugs is more pronounced under conditions when action potentials are *ab ovo* longer, while the pacing rate being just one of the factors controlling baseline APD. Although the view that the mathematical relationship between APD and I_{net} may actually cause reverse rate dependent APD changes was fully confirmed by the present results, several observations indicate that additional mechanisms may also influence the frequency dependence of APD changes. For instance, if one exclusive mechanism accounted for the reverse rate dependence, we would expect the relationship between drug-induced APD changes and baseline APD to be identical in all cases. However, such a relationship was almost linear with some drugs, while non-linear with others (e.i. effect of Bay K 8644 at longer CL). This implies that even the dependency of APD modulation on baseline APD may include components that cannot be explained by a single mechanism. Furthermore, the existence of an intrinsic dependency of APD modulation on baseline APD does not rule out the contribution of other genuinely rate dependent mechanisms, such as drug-ion channel interactions or rate dependency of ion current kinetics to determine the magnitude of the rate dependent APD change. Indeed, the magnitude of reverse rate dependency was different among the various drugs tested.

The main conclusion of the present study is that further development of selective I_{Kr} blocker drugs as potential class 3 antiarrhythmic agents are not likely to be successful.

SUMMARY

Since the SWORD (Survival With ORal D-sotalol) trial is known that class 3 antiarrhythmic agents exhibit reverse rate dependent lengthening of the action potential duration (APD) and refractery period. This indicates that changes in APD are greater at slower than at faster heart rates. Therefore in case of tachycardia when cycle lengths are pathological short and antiarrhythmic effect is most needed these agents have no proper effects on the lengthening of the repolarization and effective refractery period. However, in case of bradycardia when cycle lengths are long, the extremly prolonged repolarization can lead to formation of early afterdepolarization and life-threatening ventricular arrhythmias (tachycardia). The exact electrophysiological mechanism responsible for reverse rate dependency of class 3 antiarrhythmic drugs has not been clarified. For that reason, the detailed description of the underlying mechanism is really important, because probably it should provide a chance to develop more effective antiarrhythmic drugs.

Our experiments confirmed that reverse rate dependency is not only the property of class 3 antiarrhythmic drugs, but almost all drugs modulating action potentail show this feature. Reverse rate dependency can be considered as a general intrinsic property, which is well correlated with baseline APD and APD changes and independent of the species or preparation used, or modified ion channels. Thus we can conclude that reverse rate dependency may simply be a consequence of the inverse relationship existing between APD and net membrane current flowing during the plateau phase. The main conclusion of the present study is that the further development of selective I_{Kr} blocker drugs as potential class 3 antiarrhythmic agents are not likely to be successful. A more promising approach in the therapy of arrhythmias might be to combine distinct molecules or applying single drugs having intrinsically combined modes of action thus decrease the proarrhythmic risk of classic antiarrhythmic drugs.

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List of publications related to the dissertation

1. Bányász, T., **Bárándi, L.**, Harmati, G., Virág, L., Szentandrássy, N., Márton, I., Zaza, A., Varró, A., Nánási, P.P.: Mechanism of reverse rate-dependent action of cardioactive agents.
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