SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The beat-to-beat variability of the cardiac action potential duration

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The Examination takes place at the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen at 11 am, on March 16, 2015.

Head of the **Defense Committee**:	Prof. János Szöllősi, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1 pm, on March 16, 2015.

Introduction

It has been estimated that about half of the patients suffering from heart failure die from an arrhythmia, accounting for >500.000 deaths per year worldwide. Many mechanisms have been identified to contribute to the development of arrhythmias and the research is focused on the finding of a good predictor of the development of these arrhythmias causing sudden death. One of the electrophysiological hallmarks of heart failure is the increase in QT interval what reflects the duration of the ventricular repolarization. Prolongation of QT interval is considered a risk and an important predictor for the development of arrhythmias, in particular Torsade de Points (TdP). Nevertheless, recent studies suggest that the beat-to-beat variability is a better predictor of drug-induced torsades de pointes arrhythmias than the measurement of repolarization prolongation alone.

Beat-to-beat variability of action potential duration (also called short term variability, SV) is an intrinsic property of various *in vivo* and *in vitro* mammalian cardiac preparations including the human heart. In spite of the fact that SV is considered one of the best proarrhythmic predictors, its exact ionic mechanism is poorly understood. Involvement of many factors, such as stochastic gating of ion channels, cell-to-cell coupling, action potential duration (APD) and morphology, stimulation frequency and intracellular Ca^{2+} handling in modulation of SV have been reported, however, neither their relative contribution, nor the role of the specific cardiac ion currents have been identified in a well defined experimental model. Therefore, in absence of relevant human cellular electrophysiological data, canine ventricular myocytes were chosen to analyze experimentally the determinants of SV in the present study due to two reasons. Canine ventricular myocytes are believed to resemble human ventricular cells regarding their action potential (AP) morphology and kinetics of the underlying ion currents; and a large

mass of *in vitro* and *in vivo* experimental data on beat-to-beat variability have been obtained in this species.

Aims

Our aims were to investigate the causes and modulators of the short term beat-to-beat variability of the APD. We hypothesize that the significantly increased beat-to-beat variability is a good predictor for ventricular arrhythmias, and that this mechanism is multifactorial. Therefore we aimed to determine:

- the role of the stimulation frequency in modulating the SV
- the SV in myocytes dispersed from different regions of the left ventricle
- APD-dependence of the SV by applying electrotonic current injections
- the role of intracellular Ca^{2+} concentration in modulating the SV
- the role of inward and outward currents in modulating the SV
- and finally the role of the redox potential in modulating the SV.

Materials and methods

Isolation of single canine ventricular myocytes

Our experiments were performed using enzimatically isolated canine left ventricular myocytes. Dogs were according to protocols approved by the local ethical committee (license No: 18/2012/DEMÁB) in line with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. According to the segment perfusion technique the hearts were quickly removed and placed in Tyrode solution, followed by the cannulation of the left anterior descending coronary artery. After gradually restoring the normal external Ca^{2+} concentration, the cells were stored in Minimum Essential Medium Eagle until use.

Electrophysiological measurements

All electrophysiological measurements were performed at 37 °C. The rod-shaped viable cells showing clear striation were sedimented in a plexiglass chamber of 1 ml volume allowing continuous superfusion (at a rate of 2 ml/min) with modified Krebs solution gassed with a mixture of 95 % O_2 and 5 % CO_2 at pH = 7.4. The modified Krebs solution contained (in mM): NaCl, 128.3; $NaHCO_3$, 21.4; KCl, 4.0; $CaCl_2$, 1.8; $MgCl_2$, 0.42; and glucose 10.

Recording of action potentials

Transmembrane potentials were recorded using 3 M KCl filled sharp glass microelectrodes having tip resistance between 20 and 40 MΩ according to the technique demonstrated by Volders et al. These electrodes were connected to the input of Multiclamp 700B amplifiers. The cells were paced through the recording electrode at steady cycle length of 1 s using 1-2 ms wide rectangular current pulses. Action potentials were digitized (at 200 kHz using Digidata 1440A) and stored for later analysis.

Alteration of action potential duration

In specific experiments we aimed to alter the APD. This can be reached by applying different ion channel blockers and activators or electrotonic current injections or even by altering the stimulation frequency. In the experiments with electrotonic injections we used current pulses of various amplitudes – during the full duration of the AP except phase 0 – allowed the modification of APD within a reasonably wide range (from 20 to 500 ms). The current injection started.

Quantitative assessment of the beat-to-beat variability

Changes in SV were typically presented as Poincaré plots where 50 consecutive APD values are plotted, each against the duration of the previous AP.

The area on the Poincaré plots – covered by the consecutive APD-s are proportional to the SV. Series of 50 consecutive AP-s were analyzed to estimate SV according to an equation. The quantitative assessment of the beat-to-beat variability was achieved (beside the Poincaré plots) with the following equation:

$$SV = \frac{\sum_{i=1}(|APD_n - APD_{n-1}|)}{[n\sqrt{2}]}$$

where SV is the short term variability, APD_n and APD_{n-1} indicate the durations of the n^{th} and $n\text{-}1^{th}$ AP-s, respectively, at 90 % level of repolarization, and n denotes the number of consecutive beats analyzed.

Cell shortening measurement

Cells were field stimulated at a steady-state stimulation rate of 1 Hz using suprathreshold square wave pulses through a pair of platinum wires. Cell shortening was measured using a video-edge detector system sampling at 240 Hz. Cell shortening was expressed as a percent of initial diastolic cell length. The analogue signal was amplified, digitized and recorded with pClamp 10 software.

Statistics

Results are expressed as mean ± SEM values. Statistical significance of differences was evaluated using one-way ANOVA followed by Student's *t*-test. Differences were considered significant when *P* was less than 0.05.

Results

Transmural distribution of short term beat-to-beat variability of action potential duration

Since considerable differences are known to exist in the set and densities of ion currents, resulting in different AP morphologies, in the various layers of the ventricular wall, SV was compared in myocytes dispersed from the subepicardial

(EPI), subendocardial (ENDO) and midmyocardial (MID) regions of the left ventricle. SV was the greatest in the MID cells (2.93±0.07 ms; n=94) smallest in EPI cells (2.16±0.17 ms; n=13), and had an intermediate level in ENDO myocytes (2.53±0.16 ms; n=18). Although all these transmural differences in APD and SV were statistically significant when analyzed using ANOVA (P<0.05), they were not related to the spike-and-dome configuration of AP-s observed in EPI or MID cells. When the transient outward K^+ current (I_{to1}) was suppressed by 1 mM 4-aminopyridine in EPI cells, the "spike-and-dome" AP morphology disappeared but SV decreased slightly (control: 3.24±0.35 ms, 4-aminopyridin: 2.67±0.31 ms; n=9) instead of increasing. This concentration of 4-aminopyridine failed to modify APD significantly. The observed transmural distribution of SV was largely proportional to APD values. Therefore, the relationship between SV and APD was further studied.

The relationship between short term beat-to-beat variability and action potential duration

Altering the action potential duration by applying electrotonic current injections

To study the dependence of SV on APD, outward and inward current injections were applied in current clamp mode. The current pulses, having amplitudes varied from −600 to +70 pA, were injected throughout the full duration of the AP except phase 0. These experiments, similarly to all results discussed in the followings, were performed in midmyocardial cells. Using current pulses of various amplitudes allowed the modification of APD within a reasonably wide range (from 20 to 500 ms) in a way not related to exclusive interaction with one or another specific ion current. Current pulses having amplitude of −80 pA shortened the APD (from 213±7 ms to 138±12 ms; P<0.05) and decreased the SV (from 2.69±0.16 ms to 1.69±0.17 ms; P<0.05), while +40 pA increased the APD (to 311±19 ms; P<0.05) and SV (to 7.44±1.28 ms; P<0.05).

The results obtained by analyzing 117 data points (i.e. corresponding SV and APD values, each representing a group of 50 consecutive AP-s). The SV-APD relationship follows exponential kinetics. Similarly, an exponential relationship was observed when the current-induced changes in SV (ΔSV) and APD (ΔAPD) were analyzed. From the ΔSV-ΔAPD relation one can estimate that a change in SV caused by any given drug or intervention is greater or less than the value predicted by the concomitant change in APD (*standard curve*). The most important conclusion from these experiments is that any drug-induced change in SV must be evaluated in terms of *relative SV*, i.e. by comparing changes in SV to concomitant changes in APD.

Effect of the pacing cycle length on the short term beat-to-beat variability of action potential duration

Since the duration of a cardiac AP is strongly influenced by the frequency of stimulation, it was reasonable to study whether the frequency-dependent changes in SV are in line with the predictions of the APD changes. Both APD and SV increased with lengthening of pacing cycle length (control 1 Hz/1 sec: APD 244±8 ms, SV 3.50±0.17 ms; 0.2 Hz/5 sec: APD 308±13 ms, SV 6.33±0.42 ms; $P<0.05$, n=8). When each SV was plotted as a function of the corresponding APD value, and the exponential *standard curve* obtained from the experiments presented previously was superimposed, the experimental data gave a good fit to the curve – at least for cycle lengths longer than 0.5 s. SV values obtained at higher frequencies were larger than predicted by the APD-SV relationship indicating the possible contribution of factors other than APD at higher pacing frequencies.

Contribution of the outward ion currents to modulation of short term beat-to-beat variability of action potential duration

The role of the various ion currents in modulation of SV can be best studied by using their selective activators and inhibitors. Contribution of five K^+ currents, the I_{Kr}, the I_{Ks}, the I_{to1} and the I_{K1} was studied using specific blockers: dofetilide, HMR 1556, chromanol 293B in the presence of HMR 1556, and $BaCl_2$, respectively. The $I_{K\text{-}ATP}$ was activated by lemakalim. The I_{Kr} inhibitor dofetilide (10, 30, 100 and 300 nM) increased both APD and SV, however, its effect on SV was significantly stronger than its effect on APD (relative enhancement of SV). Similar effect could be observed with the selective I_{Ks} blocker HMR 1556 (0.5 μM), which agent caused only a small, but statistically significant lengthening of APD combined with a pronounced increase in SV. The data obtained with HMR 1556 is located above the *standard curve* in the positive ΔAPD range. This indicates that the SV-increasing effect of HMR 1556 is greater than predicted by the earlier discussed APD-SV relationship – in spite of the fact that I_{Ks} is considered to be a weak current in canine ventricular myocytes under baseline conditions. A relatively selective I_{to1}-blockade can be achieved by applying chromanol 293B in the presence of HMR 1556. Exposure of myocytes to 100 μM chromanol 293B revealed that suppression of I_{to1} decreased SV without significant alterations in APD, a result similar to previously obtained with 1 mM 4-aminopyridine. Increasing concentrations of lemakalim (0.1, 0.3, 1 and 5 μM) were used to shorten APD. Lemakalim decreased both SV and APD, but data points remained on the *standard curve* indicating that this current has little specific influence on *relative SV*. Suppression of I_{K1} with $BaCl_2$ (gradually increasing $BaCl_2$ concentration from 0.3 to 5 μM) increased APD and SV, but in this case the SV-increasing effect was weaker than predicted by the APD-SV relationship, suggesting that *relative SV* might be increased by I_{K1}. However, $BaCl_2$ caused strong triangulation of AP-s, defined as a difference between the APD_{90} and APD_{50} values. Triangulation was 47±3 ms under control conditions, which

increased to 90±11 ms in the presence of 5 μM $BaCl_2$ (P<0.05). Assuming that APD measured close to the plateau level may be more relevant to control AP repolarization than APD_{90}. Considering the corrections due to triangulation, each $BaCl_2$ data point touched the *standard curve* indicating that I_{K1} is a current largely indifferent regarding the modulation of SV.

Contribution of the inward ion currents to modulation of short term beat-to-beat variability of action potential duration

The role of I_{Na} was examined by suppression of I_{Na} using tetrodotoxin and lidocaine, or alternatively, by activation of the current by veratridine. As expected, veratridine (10, 30 and 100 nM) increased both APD and SV in a concentration-dependent manner, while both parameters were simultaneously decreased by tetrodotoxin (3 μM) and lidocaine (50 μM). Importantly, the SV-decreasing effect of I_{Na} inhibition, as well as the enhancement of SV caused by veratridine, were larger than predicted by the APD-SV relationship, congruently with an SV increasing action of I_{Na}. The other inward current under investigation was the $I_{Ca,L}$. It was blocked by 1 μM nisoldipine and enhanced by 20 nM or 200 nM of Bay K8644. Nisoldipine increased SV while APD was strongly shortened, both effects were statistically significant (control: APD 220±6 ms, SV 2.44±0.20 ms; nisoldipine: APD 116±7 ms, SV 2.96±0.27 ms; P<0.05, n=19/13). Bay K8644 increased APD significantly in 20 nM as well as 200 nM concentrations. SV was not altered by 20 nM Bay K8644, but it was increased in the presence of 200 nM Bay K8644. These changes in SV corresponded to a *relative* reduction of SV when compared to the concomitant lengthening of APD. In other words, SV was strongly diminished by $I_{Ca,L}$.

Offsetting the effects of APD changes

The best approach to separate the contribution of a specific ion current to modulating SV from the effect of the concomitant APD-change is to apply an electrical or pharmacological compensation. The former strategy was used where the APD-lengthening effects of 0.1 μM dofetilide, 5 μM $BaCl_2$, 0.1 μM veratridine and 0.2 μM Bay K8644 were offset using outward current pulses with constant, properly chosen amplitude. Similarly, the APD-shortening effects of 3 μM tetrodotoxin and 1 μM nisoldipine were compensated using inward current pulses. After full compensation for the dofetilide-induced lengthening of APD a significant elevation of SV persisted (control: 2.37±0.19 ms; dofetilide+tone: 2.68±0.29 ms; $P<0.05$, n=7), while in the case of $BaCl_2$ the enhancement of SV was eliminated by restoring the original value of APD (control: 2.31±0.20 ms; $BaCl_2$+tone: 1.97±0.16 ms; $P<0.05$, n=8). More specifically, SV, measured after compensation for the barium-induced lengthening of APD, fell below the control level of SV. However, applying the argumentation used previously, based on the barium-induced triangulation of AP-s, APD_{50} was overcompensated by the applied outward current. This is in line with our previously discussed finding, i.e. suggesting that I_{K1} is really an indifferent current regarding its possible influence on SV. Similarly to results obtained with dofetilide, SV remained elevated in the presence of veratridine even if APD was fully compensated (control: 2.25±0.19 ms; veratridine+tone: 3.95±0.24 ms; $P<0.05$, n=14) – in contrast to Bay K8644, which resulted in a subnormal level of SV after compensation for the APD-changes (control: 2.18±0.19 ms; Bay K8644+tone: 1.71±0.16 ms; $P<0.05$, n=10). When I_{Na} was blocked by tetrodotoxin, the SV values were lower than control following compensation of APD (control: 2.75±0.29 ms; tetrodotoxin+tone: 2.17±0.28 ms; $P<0.05$, n=8), while in the presence of the $I_{Ca,L}$ blocker nisoldipine the drug-induced elevation of SV was further increased by the inward current pulse applied for offsetting the APD-changes (control: 2.68±0.20 ms; nisoldipine+tone: 5.38±0.59 ms; $P<0.05$, n=10).

Since one may argue that electrical compensation of APD-changes may shift the membrane potential to one or another direction, pharmacological compensation was also used in some experiments. In this case the APD-lengthening effects of dofetilide and veratridine were compensated by lemakalim (using properly chosen concentrations in each experiment), while the APD-shortening effects of tetrodotoxin and nisoldipine were compensated by $BaCl_2$. Although these compensations resulted in full restoration of the initial APD_{90} values, SV remained elevated in the presence of dofetilide and veratridine, became higher than control in nisoldipine and lower than control in tetrodotoxin. In summary, electrical and pharmacological compensation strategies yielded largely identical results corroborating the specific SV-lowering effects of I_{Kr} and $I_{Ca,L}$, as well as the SV-increasing effects of I_{Na}. It must be emphasized, however, that the most dramatic effect was observed with nisoldipine, independently of the way of compensation, highlighting the pivotal role of $I_{Ca,L}$ in controlling SV.

Effect of intracellular calcium homeostasis on modulating the short term beat-to-beat variability of action potential duration

Role of intracellular calcium concentration in modulating the short term beat-to-beat variability of action potential duration

There are various manipulations suitable for modulation of $[Ca^{2+}]_i$ experimentally – the majority of them involves interactions with SR Ca^{2+} handling. Instead of these, the simplest strategy was followed in the present study – based on the assumption that if the cell is loaded directly with Ca^{2+}, it must increase $[Ca^{2+}]_i$, and conversely, $[Ca^{2+}]_i$ must be reduced by an intracellularly applied Ca^{2+}-chelator. Exposure of the cells to the Ca^{2+}-ionophore A23187 (1 μM for 25 min) significantly shortened (from 207±4 ms to 170±5 ms; $P<0.05$, n=14), while loading the cells with the cell-permeant acetoxy-methylesther form of the Ca^{2+} chelator BAPTA (using 5 μM BAPTA-AM for 25 min) strongly lengthened APD (from 218±8 ms to 297±15 ms; $P<0.05$, n=17), although SV was not modified

significantly by these interventions. However, A23187 increased, while BAPTA-AM decreased the *relative SV* when it was compared to the *standard curve* obtained with electrotonic current pulses.

The effect of BAPTA-AM was further investigated by combining current injections with BAPTA-AM pretreatment. In these experiments AP-s were prolonged or shortened with the application of appropriately tailored depolarizing or hyperpolarizing pulses, respectively. Thus the SV-APD relationship could be determined in the presence of BAPTA-AM and compared to that obtained under control conditions. APD after BAPTA-AM perfusion was longer at any current injections compared to that of the control (−80 pA: APD 190±7 ms, SV 1.86±0.12 ms; +40 pA: 414±25 ms, SV 9.14±2.46 ms). According to our results *relative SV* is reduced by BAPTA-AM at any APD value, however, this reduction was greater and more prominent at longer APDs.

Contribution of sarcoplasmic reticular Ca^{2+} release to modulation of short term beat-to-beat variability of action potential duration

Contribution of transient changes of $[Ca^{2+}]_i$ due to Ca^{2+} released from the sarcoplasmic reticulum (SR) was studied using ryanodine and cyclopiazonic acid. The former blocks Ca^{2+} release from the SR at 10 μM concentration by decreasing the open probability of the Ca^{2+} release channel in the SR membrane. Cyclopiazonic acid is a selective inhibitor of the SR Ca^{2+} pump resulting in a depletion of the SR Ca^{2+} release pool. Application of these agents resulted in a comparable reduction of *relative SV*. Ryanodine caused a transient decrease in SV with no significant change in APD (from 2.92±0.12 ms to 2.37±0.19 ms; $P<0.05$, n=8), while 1 μM cyclopiazonic acid decreased SV significantly (from 3.87±0.29 ms to 3.29±0.29 ms; $P<0.05$, n=9), accompanied with a small, statistically not significant reduction of APD. These results clearly indicate that systolic Ca^{2+} release, experienced by the surface membrane, is the relevant signal for SV modulation.

Role of the Na+-Ca2+ exchange in modulating the short term beat-to-beat variability of action potential duration

The influence of the Na^{+}-Ca^{2+} exchanger (NCX) on SV was studied after inhibition of the exchanger by SEA0400. This agent is thought to be a selective blocker of NCX, when applied at a sufficiently low concentration of 300 nM. Exposure of the cells to 300 nM SEA0400 for 25 min increased SV (from 2.53±0.16 ms to 2.77±0.14 ms; P<0.05, n=7) and decreased APD significantly (from 235±10 ms to 206±13 ms; P<0.05, n=7), clearly resulting in a marked enhancement of *relative SV*.

Effects of BAPTA-AM, A23187, ryanodine and cyclopiazonic acid on cell shortening

Effects of BAPTA-AM, A23187, ryanodine and cyclopiazonic acid were also studied on unloaded cell shortening. Drugs were superfused after reaching steady-state amplitudes of shortening. Shortening of the cells was suppressed by all of the drugs studied. While in the case of BAPTA-AM, ryanodine and cyclopiazonic acid the decreased cell length approximated the diastolic level, indicating a suppressed Ca^{2+} release, the enveloping curve of the cell shortening was close to the systolic level in the presence of A23187. This is in line with the Ca^{2+} accumulation expected in the presence of a Ca^{2+} ionophore.

Effect of β-adrenergic stimulation on the short term beat-to-beat variability of action potential duration

Summarizing the results above it could be concluded that two currents, namely $I_{Ca,L}$ and I_{Ks}, were very effective in reducing the *relative SV*. In this case, isoproterenol (ISO) is expected to decrease SV markedly, since this drug is known to increase both $I_{Ca,L}$ and I_{Ks} simultaneously. 10 nM ISO caused a small, although statistically significant shortening in APD at 1 Hz (from 219±6 ms to 195±7 ms; P<0.05, n=13), which was accompanied with a robust decrease in SV (from

2.31±0.12 ms to 1.43±0.11 ms; $P<0.05$, n=13), which was much larger than predicted by the APD-SV relationship. Indeed, the lowest SV could be observed at close to normal APD values in the presence of 10 nM ISO. Since ISO is known to increase $[Ca^{2+}]_i$, and changes in $[Ca^{2+}]_i$ were shown to influence *relative SV*, the effects of ISO were studied also after the exposure to 5 μM BAPTA-AM for 25 min. As seen previously, pretreatment with BAPTA-AM lengthened APD markedly without significantly affecting SV. In the presence of BAPTA-AM SV was decreased and APD was shortened by ISO similarly to results observed without BAPTA-AM. The effect of ISO on *relative* SV was largely similar in the presence and absence of BAPTA-AM, indicating that the effect of ISO on SV was not related to the concomitant changes in $[Ca^{2+}]_i$, it was rather caused by the ISO-induced augmentation of $I_{Ca,L}$ and I_{Ks}.

The effect of 10 nM ISO was studied also at various pacing cycle lengths. In these experiments SV was determined by analyzing 10 consecutive AP-s only in order to limit the duration of the measurement. Although both the ISO-induced shortening of APD and reduction of SV increased with increasing the cycle length of stimulation, the *relative SV* progressively decreased at longer cycle lengths.

Impact of tissue redox potential on the short term beat-to-beat variability of action potential duration

Exposure of myocytes to reductive environment had little effect on AP morphology, including the APD. In contrast, SV was significantly decreased by the reductive cocktail (control: 2.81±0.30 ms; reductive cocktail: 2.12±0.15 ms; $P<0.05$, n=12), containing 1 mM DL-dithiothreitol, 1 mM reduced L-glutathione and 1 mM L-ascorbic acid. This effect of the reductive cocktail developed rapidly, the maximal reduction in SV was achieved within 6 min and SV failed to change afterwards. In order to offset the small and variable (statistically not significant) changes of APD developing in the reductive cocktail, *relative SV* was determined by plotting a given SV value as a function of the corresponding APD, and *relative*

SV changes were estimated by plotting ΔSV against the ΔAPD. In order to eliminate the possible contribution of any concomitant change in APD, these data were compared to the *standard curve*. Data points appearing below the *standard curve* indicate reduction of *relative SV* in response of the reductive environment.

In contrast to reduction, oxidation caused profound changes in AP morphology. APD was progressively increased in the presence of the oxidant 10 μM H_2O_2, and typically after 10-15 min of H_2O_2 superfusion early afterdepolarizations (EAD) developed. Since both APD and SV values were increased significantly by H_2O_2 (control: APD 214±8 ms, SV 2.57±0.24 ms; 9. min H_2O_2: APD 265±10 ms, SV 9.46±1.43 ms; $P<0.05$, n=10), *relative SV* was determined. *Relative SV* was increased markedly by the oxidative shift in the redox potential. Elevation of SV was not accompanied by development of EAD-s within the initial 9 min exposure to H_2O_2.

Finally, we aimed to show that these drastic effects of H_2O_2 superfusion are really consequences of redox potential changes. In the next series of experiments, therefore, 10 μM H_2O_2 was applied in the presence of the reductive cocktail following a 5 min period of pretreatment. The elevation of *relative SV*, observed previously in H_2O_2, was fully prevented by the reductive environment having a sufficiently high redox buffering capacity. This was true in spite of the fact that both SV and APD were increased by the H_2O_2 exposure (reductive cocktail: APD 203±11 ms, SV 2.37±0.15 ms; H_2O_2+reductive cocktail: APD 231±13 ms, SV 3.14±0.36 ms; $P<0.05$, n=6).

Discussion

Relationship between action potential duration and beat-to-beat variability

According to the conventional interpretation of beat-to-beat variability, it is due to the stochastic behavior of ion channel gating. Without questioning the contribution of this mechanism, here we propose another (probably more relevant) one to explain the observed changes in SV. Physiological control of APD is based on the following well-known negative feed-back regulation scheme: prolongation of APD (e.g. due to the enhancement of an inward current) results in elevation of the plateau, which in turn accelerates the activation of I_{Kr} and I_{Ks} leading finally to a shortening of APD. Accordingly, those currents which are known to be critical members of this feed-back loop – namely $I_{Ca,L}$, I_{Kr} and I_{Ks} – are expected to decrease the variability of APD, and their inhibition may have an opposite effect. This interpretation provides some explanation for the following question: why $I_{Ca,L}$ decreases while late I_{Na} increases APD. This is not evident since both currents are inwardly directed, tending to increase APD together with its variability. The crucial difference between $I_{Ca,L}$ and late I_{Na} is that the former is active mainly during the initial part of the plateau, while the latter is dominant during the late plateau. As a consequence, $I_{Ca,L}$ has a chance to shift the early plateau upwards allowing for faster and stronger activation of I_{Kr} and I_{Ks}, while in the case of late I_{Na} these changes develop later in time and in a less pronounced way. In line with this, the SV-increasing effect of late I_{Na} is strong because its activation overlaps the late plateau, where the membrane resistance is the highest, consequently, a relatively small inward shift in the net membrane current may result in a large prolongation of APD. This gives also the physical basis of the exponential relationship between SV and APD, as was predicted by the simulations of Heijman et al. and confirmed experimentally by the present work.

Our results regarding the effects of the stimulation cycle length on SV is similar to those of Johnson et al., both studies showing an elevation of SV with increasing the cycle length. It might be tempting to associate these frequency-dependent changes in SV with the corresponding changes in the amplitude of specific ion currents. Since $I_{Ca,L}$ is known to increase, while I_{Ks} is to decrease with increasing the cycle length of stimulation, the value of this approach seems to be limited. However, we have shown also that at cycle lengths of 700 ms or longer the changes in SV was likely due to the concomitant alterations of APD, since no change in *relative SV* could be observed within this frequency range. Specific cycle length-dependent changes in SV could be observed only at the shortest cycle lengths, where $[Ca^{2+}]_i$ could probably be elevated. Indeed, manipulation of $[Ca^{2+}]_i$ resulted in the expected change in the *relative SV*, which increased and decreased together with the changes in $[Ca^{2+}]_i$. In line with this assumption, the ISO-induced reduction of *relative SV* also increased with increasing the cycle length of stimulation. Accordingly, the ISO-induced reduction of SV was not significant at the shortest cycle length of 0.3 s, while it became progressively dominant at longer cycle lengths (including the range between 0.5 and 1 s, corresponding to the physiological heart rate in humans). It can be speculated that the ISO-induced reduction of relative SV could partially be offset by the elevated $[Ca^{2+}]_i$ at faster driving rates.

Role of ion currents in modulating the beat-to-beat variability

The main goal of the present study was to separate SV changes related to inhibition of a specific ion current from those attributable to concomitant changes in APD. This approach allows for separation of ion currents to APD-stabilizing, therefore potentially antiarrhythmic currents (ones *decrease relative SV*) and to those which *increase relative SV*. Members of this latter group result in instability of APD, therefore they can be considered potentially arrhythmogenic. Using these categories based on predictions of arrhythmia incidence, 3 currents could be

identified as APD-stabilizer: $I_{Ca,L}$, I_{Ks} and I_{Kr}. I_{Na} and I_{to} were found to increase instability of APD, while I_{K1} and $I_{K\text{-}ATP}$ appeared to be indifferent. These results provide an essentially new interpretation of beat-to-beat variability suggesting that the well-known negative feed-back regulation of APD may be an important factor of SV-modulation.

Effects of several ion channel modifiers (including dofetilide, HMR 1556, ATX-II and isoproterenol in canine, while tetrodotoxin and intracellular EGTA in guinea pig ventricular cells) on the magnitude of beat-to-beat variability have been extensively studied, however, the changes in SV were not correlated by the investigators with the concomitant APD changes. The first approach to make such a distinction was the recent study of Heijman et al., although it was an *in silico* analysis. Present results, however, have confirmed many of their predictions: I_{Na} and I_{Kr} have great impact on SV, I_{Kr} is an APD-stabilizing, while I_{Na} and I_{to} are APD-unstabilizing currents. I_{Ks} was estimated also as an APD-stabilizing current (having larger impact on SV than on APD) - in line with our observations, but only our analysis, based on comparison of ΔSV-ΔAPD changes, was able to highlight the importance of this interaction in the case of I_{Ks}. The largest difference between our and their results was found in the role of $I_{Ca,L}$. Although $I_{Ca,L}$ was considered to be APD-stabilizing by both studies, it was the most important modulator in our experiments, as indicated by the results obtained with nisoldipine and Bay K8644 – in contrast to the moderate effect on SV proposed by the study of Heijman et al.

In addition to APD, the morphology of AP was also suggested to be an important determinant of SV. Heijman et al. simulated square-like AP configuration by decreasing both $I_{Ca,L}$ and I_{to}, while triangulation was achieved by strongly reducing I_{K1}. SV was dramatically elevated when it was simulated for a square-like AP, on the contrary, triangulation decreased *relative* SV. Indeed, characteristic changes in AP morphology can be attributed to modifications of many cardiac ion currents, e.g. suppression of $I_{Ca,L}$ by nisoldipine results in plateau depression and square-like morphology. Similarly, suppression of I_{K1} caused

triangulation of AP-s, i.e. smaller increase of APD_{50} than that of APD_{90}. Since the plateau level is critical in the negative feed-back regulation of APD, its duration is overestimated by APD_{90} in case of triangulation. This is why I_{K1} can not be considered as a potentially proarrhythmic current in dogs in spite of the reduction of *relative SV* observed in $BaCl_2$.

Although modification of SV observed in these examples is likely mediated by a change in AP morphology, this is not always the case. For example, exposure of EPI myocytes to 4-aminopyridine converted AP morphology from EPI to ENDO, but SV was not increased accordingly. Furthermore, SV was reduced by ISO very effectively without causing triangulation.

Role of intracellular calcium concentration in modulating the beat-to-beat variability

We have intentionally chosen the simplest way to manipulate $[Ca^{2+}]_i$, since both chelation of $[Ca^{2+}]_i$ and increasing the Ca^{2+} entry using a Ca^{2+} ionophore are expected to yield steady changes in $[Ca^{2+}]_i$, relatively independent of the cardiac cycle. This allowed a qualitative prediction of SV changes associated with variation of $[Ca^{2+}]_i$, but is obviously not suitable for modeling the consequences of dynamic $[Ca^{2+}]_i$ changes occurring when transports between the SR and the cytosol are active.

In the present study we have shown that *relative SV* was increased by elevation of $[Ca^{2+}]_i$ and decreased by its reduction. This was true practically independently of the actual experimental conditions. Best demonstration of this finding, where chelation of $[Ca^{2+}]_i$ by BAPTA-AM decreased *relative SV* in all cases. This reduction in *relative SV* was mainly due to an actual decrease in SV when BAPTA-AM was applied following I_{Kr} blockade by dofetilide or E-4031. Under these condition APD was shortened by BAPTA-AM. On the other hand, when APD was lengthened by veratridine or Bay K8644 (due to enhancement of $I_{Na\text{-}late}$ and $I_{Ca,L}$, respectively), as well as under control conditions, *relative SV* was

decreased by BAPTA-AM due to the marked prolongation of APD. The BAPTA-AM induced prolongation was likely the consequence of a direct blocking effect of BAPTA-AM on I_{Kr}. Although the underlying ionic mechanism was basically different under these conditions (i.e. the set of operating Ca^{2+}-sensitive ion channels were likely different), reduction of $[Ca^{2+}]_i$ decreased *relative SV* uniformly.

In line with this approach, SEA0400, the potent inhibitor of NCX strongly increased *relative* SV. NCX is believed to be the main mechanism of Ca^{2+} extrusion from the intracellular space in cardiac cells. Suppression of this component of Ca^{2+} elimination is expected to increase $[Ca^{2+}]_i$ beneath the sarcolemma, which effect was well documented in rat myocytes. Although no significant increase in $[Ca^{2+}]_i$ could be detected in canine cells on exposure to SEA0400 when measuring $[Ca^{2+}]_i$ in the bulk phase of the cytosol, it is likely that suppression of NCX results in an elevated $[Ca^{2+}]_i$ in the submembrane compartment of canine myocytes as well. Indeed, selective suppression of NCX by SEA0400 increased SV while APD was reduced. Both changes were likely consequences of the elevated submembrane Ca^{2+} concentration. It is worthy of note that under markedly different experimental conditions (after prolongation of APD and development of EAD-s by dofetilide) 1 μM SEA0400 decreased the short term variability of APD and suppressed EAD-s, while the elevated amplitude of $[Ca^{2+}]_i$ transient was also decreased by SEA0400. Although the effects of NCX inhibitors on $[Ca^{2+}]_i$ are strongly depend on experimental conditions (here $[Ca^{2+}]_i$ was decreased by SEA0400 - in contrast to our results obtained under baseline conditions), reduction of $[Ca^{2+}]_i$ was accompanied by a decreased SV, supporting further our conclusions.

Role of sarcoplasmic reticular calcium release in modulating the beat-to-beat variability

More importantly, the contribution of the SR-related $[Ca^{2+}]_i$ release seems to be a dominant factor in Ca^{2+}-sensitive modulation of SV. Ryanodine, the blocker of the SR Ca^{2+} release channel decreased SV directly. Cyclopiazonic acid, the selective inhibitor of the SERCA Ca^{2+} pump resulting in a depletion of the SR Ca^{2+} release pool caused similar effect. Both agents are known to decrease the amplitude of the intracellular Ca^{2+} transient in cardiac cells, and in line with these results, they decreased unloaded cell shortening in our experiments. Although the time-dependent pattern of changes in SV and APD were different, the resultant reduction in *relative SV* was similar with both drugs. These observations are in accordance with the results of Johnson et al. demonstrating the increment of SV during diastolic spontaneous $[Ca^{2+}]_i$ release induced by β-adrenergic stimulation. The important role of $[Ca^{2+}]_i$ transients in modulation of SV supports the view that it is manifested in fact by the Ca^{2+}-sensitive ion channels in the surface membrane, which are known to directly experience $[Ca^{2+}]_i$ changes in the narrow submembrane compartment, mentioned also as fuzzy space.

Regarding the underlying mechanisms (i.e. which Ca^{2+} sensitive ion current may be responsible for the Ca^{2+} sensitivity of SV), it has been shown that there are 3 ion currents in canine myocytes which have deep impact on beat-to-beat variability, these are: late I_{Na}, I_{Kr} and $I_{Ca,L}$. Although activation of I_{Na} or suppression of I_{Kr} were reported to increase SV drastically, these alterations cannot be easily deduced from elevation of $[Ca^{2+}]_i$. The third current is $I_{Ca,L}$, which is suppressed in amplitude by high $[Ca^{2+}]_i$ due to its Ca^{2+} dependent inactivation. Indeed, relative SV was reduced by Bay K8644 and increased by nisoldipine, indicating that $I_{Ca,L}$ is an important regulator of SV. In conclusion, the major effect of $[Ca^{2+}]_i$ on SV is likely to occur *via* reduction of $I_{Ca,L}$ by enhancing its Ca^{2+} dependent inactivation, however, minor contribution of other Ca^{2+} sensitive ion

currents, such as I_{NCX}, I_{Cl} and a variety of Ca^{2+} sensitive K^+ currents, cannot fully be ruled out.

Role of tissue redox potential in modulating the beat-to-beat variability

In the present study we have shown that relative SV was decreased by applying a reductive environment, while shifting the redox potential to oxidative direction strongly increased relative SV. This was accompanied by development of EAD-s following the 10-15 min of H_2O_2 superfusion. These effects of H_2O_2 could be prevented by application of a reductive cocktail. Since similar changes are known to occur under conditions of oxidative stress, it is plausible to assume that increases in beat-to-beat variability of APD, caused by an oxidative shift in the tissue redox potential, may contribute to the increased arrhythmia incidence observed in cases of ischemia/reperfusion injury.

Regarding the possible underlying mechanisms, we can only speculate in the absence of relevant voltage clamp data. Effects of redox potential changes on cardiac ion currents display large variation according to the experimental conditions and the species studied. $I_{Ca,L}$ was markedly increased by oxidative challenges in the majority of cardiac cells, including canine, rabbit and rat ventricular myocytes, similarly to HEK cells expressing the pore forming α_{1C} subunits of human L-type Ca^{2+} channels, while $I_{Ca,L}$ was decreased in guinea pig myocytes in response to oxidative stress. There was no change in I_{K1} amplitude in rabbit and guinea pig, but the other inwardly rectifying K^+ current, the ATP-sensitive K^+ current ($I_{K\text{-}ATP}$), was enhanced by oxidation in the latter species. I_{Kr} is likely to be enhanced by oxidation, since exposure of HERG channels – expressed in CHO cells – to H_2O_2 increased the amplitude of I_{Kr}. These results, however, cannot explain the increased APD variability observed in the presence of H_2O_2, since both $I_{Ca,L}$ and I_{Kr} were found to decrease SV in canine ventricular cells. Similar results were obtained in computer simulations. I_{Ks} was decreased by oxidative free radicals in guinea pig ventricular cells and this current was also

shown to decrease SV in canine myocytes and *in silico* models. However, the limited contribution of I_{Ks} to ventricular repolarization under baseline conditions in dogs may question the role of I_{Ks}. Similarly, relative SV was not much affected by either I_{K1} or $I_{K\text{-}ATP}$ in canine myocytes. The only ion current which may have a direct role in mediating the influence of redox changes on SV in dogs is I_{Na}. Amplitude of this current was increased by H_2O_2 in rabbit ventricular cells due to the removal of its fast inactivation, and I_{Na} was shown to increase SV in dogs as well as *in silico*. More importantly, elevation of $[Na^+]_i$ is known to be converted to intracellular Ca^{2+} accumulation. Indeed, accumulation of cytosolic Ca^{2+} was observed with H_2O_2 in rabbit ventricular cells, which shift has been reported to increase the beat-to-beat variability of APD. The casual role of the elevated $[Ca^{2+}]_i$ in the increased variability is further supported by the documented actions of oxidative shifts resulting in elevation of $[Ca^{2+}]_i$. Several redox sensitive mechanisms were shown to increase $[Ca^{2+}]_i$ during oxidative stress, including the higher open probability of ryanodine receptor, the increased amplitude of $I_{Ca,L}$ accompanied by an enhanced Na^+/Ca^{2+} exchange current. All these changes are known to increase beat-to-beat variability and evoke afterdepolarizations and therefore might account for the actions of the oxidative environment on beat-to-beat variability.

Clinical implications

In addition to the better understanding of the mechanism of beat-to-beat variability, there is one – quite annoying – practical implication of the present work. We have shown that I_{Kr}, I_{Ks} and $I_{Ca,L}$ act to reduce short term variability. These are the currents being generally suppressed by class 3 and 4 antiarrhythmics, respectively. Furthermore, 10 nM ISO was the most effective agent to diminish *relative SV* out of all drugs tested. Class 2 antiarrhythmics, which are beta-receptor blockers, are expected to suppress this apparently beneficial adrenergic activity. Although we do not intend to suggest that adrenergic

activation itself would be antiarrhythmic, it must be clearly seen that it has antiarrhythmic properties as well. Putting together, almost all of the presently applied antiarrhythmic agents (including the less side-effect carrying beta-blockers) may potentially increase the beat-to-beat variability of action potential duration limiting this way their antiarrhythmic potencies.

It is generally accepted that elevation of $[Ca^{2+}]_i$ is highly proarrhythmic. High $[Ca^{2+}]_i$ -induced arrhythmias are generated mainly by two mechanisms: elevation of $[Ca^{2+}]_i$ causes uncoupling of myocytes due to closure of gap junctions, resulting in increased longitudinal resistance, slower conduction, and ultimately re-entry. The second - not less important - mechanism is development of delayed afterdepolarizations, mediated by Ca^{2+}-overloaded SR. Based on the present results a third potential Ca^{2+} dependent proarrhythmic mechanism has to be considered: the increased beat-to-beat variability of APD. Further studies are required to assess the actual significance of the increased beat-to-beat variability, induced by high $[Ca^{2+}]_i$, in arrhythmogenesis.

Summary

Recent studies suggest the short term beat-to-beat variability (SV) of action potential duration (APD) as a novel method for predicting imminent cardiac arrhythmias, although the underlying mechanisms are still not clear. The goal of our experiments were to determine the role of the major cardiac ion currents, APD, stimulation frequency, redox potential and changes in the intracellular calcium concentration on the magnitude of SV. Our aim was also to study the transmural heterogeneity of SV.

Series of action potentials were recorded from isolated canine ventricular cardiomyocytes using conventional microelectrode technique. SV was quantitatively described by constructing Poincaré plots from 50 consecutive APD-s.

SV was significantly larger on midmyocardial cells compared to subepicardial and subendocardial cells. On midmyocardial cells SV was an exponential function of APD, when APD was modified by current injections. Drug effects were characterized as *relative SV* changes by comparing the drug-induced changes in SV to those in APD according to the exponential function obtained with current pulses. *Relative SV* was increased by dofetilide, HMR 1556, nisoldipine and veratridine, while it was reduced by Bay K8644, tetrodotoxin, lidocaine and isoproterenol. *Relative SV* was also increased by increasing the stimulation frequency and intracellular calcium concentration. Contribution of transient changes of intracellular calcium concentration due to calcium released from the SR was an important contributor to this process. We found a significant effect of redox potential on SV, since the reductive environment decreased, while the oxidative environment increased the *relative SV*.

We conclude that drug-induced effects on SV should be evaluated in relation with the concomitant changes in APD. In summary, *relative SV* is decreased by ion currents involved in the negative feedback regulation of APD ($I_{Ca,L}$, I_{Ks} and I_{Kr}), while it is increased by I_{Na} and I_{to}. It is concluded that elevation of intracellular calcium concentration increases *relative SV* significantly. More importantly, intracellular calcium concentration released from the sarcoplasmic reticulum is an important component of this effect. Stimulation frequency and alterations in the redox potential are also relevant SV modulating factors.

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List of publications related to the dissertation

1. Magyar, J., Bányász, T., Szentandrássy, N., **Kistamás, K.**, Nánási, P.P., Satin, J.: Role of Gap Junction Channel in the Development of Beat-to-Beat Action Potential Repolarization Variability and Arrhythmias.
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