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The role of intracellular Ca^{2+}-concentration in regulating cardiac ion currents

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The Examination takes place at the Library of Department of Physiology, Faculty of Medicine, University of Debrecen at 11 am, on 4th of December 2019.

Head of the **Defense Committee**: János Szöllősi, PhD, DSc, MHAS

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1 pm, on 4th of December 2019.

Introduction

General considerations

The blood in our body is kept in circulation by our hearts for a lifetime. The heart is an electromechanical pump, with spontaneous rhythmic action potentials (APs) alternating between contraction and relaxation. A large proportion of the heart's mass is made up of atrial and ventricular myocytes, which generate contraction force and perform pump function ("working muscle").

AP of ventricular myocytes is produced by a combination of several ion currents. These ion currents are activated in different phases of the AP and contribute to its formation to varying degrees.

In basal unactivated cells, the concentration of free Ca^{2+} in the plasma varies from 10 to 100 nM. Elevations in Ca^{2+} may induce differential, even parallel, processes that lead to the activation of many cellular functions, such as myocardial contracture. The intensity of myocardial contraction depends, within certain limits, on the magnitude of the Ca^{2+} signal and the increase in the Ca^{2+} level of the sarcoplasm. Higher levels of $[Ca^{2+}]_i$ cause an increase in contractile force.

In parallel with changes in membrane voltage, calcium oscillation of the calcium level can be observed, which has a direct, immediate effect on calcium-dependent currents. A wide range of ion currents in the heart is known to be Ca^{2+} dependent, including Na^+, Ca^{2+}, K^+, Cl^-, Na^+/Ca^{2+} exchanger, and non-specific cation currents. It is important to understand the physiological and pathophysiological function of calcium-dependent ion currents.

In cellular electrophysiological experiments, intracellular calcium concentration is often buffered to study Ca^{2+}-dependent ion currents. This is usually done so that changes in intracellular Ca^{2+}-dependent processes do not affect our measurement results.

In this dissertation, we aimed to investigate the properties of two calcium-dependent ion currents, which have been less well characterized in ventricular myocardium, using their specific inhibitors. One of these currents is the Ca^{2+}-dependent chloride current ($I_{Cl(Ca)}$) and the other is the Transient Receptor Potential Melastatin 4 (TRPM4) current.

Action potential and ionic currents of ventricular myocytes

There is a potential difference between the two sides of the living cell membrane, the intracellular space being more negative compared to the extracellular space. The resting membrane potential can vary between −50 and −90 mV, depending on the cell type. The resting membrane potential can be measured by electrophysiological methods and calculated using the Goldman-Hodgkin-Katz equation.

AP of ventricular myocytes is produced by a combination of several ion currents. These ion currents are activated in different phases of the AP and contribute to its formation to varying degrees.

During AP phase 0, fast, time- and voltage-dependent Na^+ channels are activated, which creates the AP's rising phase. Following depolarization by Na^+ channels, the channels responsible for certain currents are activated or inactivated. The high K^+ conductance (inward rectifier potassium current, I_{K1}) responsible for maintaining the resting membrane potential is reduced by inward rectification, making it impossible to generate a repolarizing K^+ current during phase zero.

Phase 1 is a rapid, short-term partial repolarization generated by transient outward current. It has a 4-aminopyridine sensitive component (transient outward potassium current, I_{to1}) and a Ca^{2+} -dependent chloride current component (transient outward chloride current, I_{to2}). During Phase 2, L-type calcium current ($I_{Ca,\,L}$) and late potassium currents are activated. During the plateau phase, there is no significant net measurable membrane current and repolarization due to the rapid inactivation of Ito and the slow activation of various repolarizing K^+ channels.

During phase 3, $I_{Ca,\,L}$ is inactivated and several potassium currents are activated in parallel, gradually. Important ion currents at this stage of the AP are the slow and fast component of the delayed rectifier potassium current (I_{Ks}/I_{Kr}) and finally the I_{K1} previously involved in terminal repolarization.

Phase 4 is the electrical diastole of non-excited ventricular myocytes, maintained by high I_{K1} conductance, equivalent to resting membrane potential of −80 mV.

Ventricular AP may be involved in several inward (e.g. TRPM4 currents) and outward (e.g. Ca^{2+} -dependent chloride currents) ion currents whose physiological function is less well characterized, but has been shown to be pathophysiologically relevant.

Spatial and temporal heterogeneity of ventricular repolarization

Cardiac AP varies between different regions of the working muscle. The spatial inhomogeneity of repolarization includes the apico-basal gradient of repolarization and the transmural dispersion, as well as the different repolarization times between the two chambers. The so-called peak towards the heart peak The action potential of the "apical" areas of the myocardium is shorter than the action potential towards the annulus fibrosus. In the "base" section. This can be caused by different rates of different potassium currents. In addition to apico-basal inhomogeneity, transmural AP inhomogeneity can also be observed. Ventricular myocardium is subdivided into three subepicardial (EPI), subendocardial (ENDO) and midmyocardial (MID) regions in terms of AP shape. The epicardial cells are called so-called. They have a "spike and dome" configuration as a result of the explicit phase 1 repolarization. Midmiocardial or M cells have less early repolarization, while endocardial cells have the smallest amount. The duration of action potentials (APDs) also varies in different layers of the chamber wall. The action potential of MID cells is longest, while that of EPI cells is short. Transmural abnormalities can be caused by varying densities of Ito and re-opening of calcium channels on EPI cells. Regional heterogeneity affects the shape of the ECG waves, whereas the increase in heterogeneity due to reentry formation is arrhythmogenic.

In the heart with a constant heart rate, the duration of successive beats varies from beats to beats, known as "beat-to-beat" variability, also known as short-term variability (SV). A number of factors are attributed to SV creation, such as: stochastic behavior of ion channels, pacing frequency and pharmacological effects, shape and duration of AP, ion channel density, or intracellular Ca 2+ control are involved in its formation.

Prolongation of the QT interval is considered a risk factor for cardiac arrhythmias, as it may promote the development of early post-depolarization (EADs) and torsades de pointes ventricular tachycardia (TdP). SV can be observed in the temporal and spatial variability of APs, in local activation and recovery intervals, or in the variability of the QT interval of the surface ECG. However, the increase in QT interval short-term variability has a higher predictive value than the prolongation of the absolute QT interval.

Calcium homeostasis of cardiac muscle cells

To study a Ca^{2+} -dependent ion current in a physiological environment, we need to be aware of changes in intracellular Ca^{2+} concentration during the cardiac cycle.

The excitation-contraction relationship of the heart muscle is the conversion of the electrical signal into a mechanical response. Ca^{2+} is essential for cellular electrical activity and

contraction, the latter being completely dependent on the Ca^{2+} concentration in the environment. The amount of Ca^{2+} released and then removed during the cardiac cycle must be the same, otherwise the cardiac muscle would be overloaded or depleted by Ca^{2+}.

The total Ca^{2+} signal that triggers the contraction is approx. 30% comes from extracellular fluid and about 70% from the sarcoplasmic reticulum (SR). During the AP plateau phase, after depolarization, Ca^{2+} enters the cardiomyocytes with $I_{Ca,\ L}$ and reverse Na^{+}/Ca^{2+} exchange currents (I_{NCX}), where it releases more Ca^{2+} from the SR via the ryanodine receptors. The phenomenon is called Ca^{2+} -induced Ca^{2+} release. The transient increase and then decrease in intracellular Ca^{2+} concentration is Ca^{2+} transient.

$I_{Ca,L}$ is activated by depolarization and then inactivated in a time- and Ca^{2+}-dependent manner. In addition to Ca^{2+} entering the cell through the ion channel, release of Ca^{2+} from SR also contributes to Ca^{2+} -dependent inactivation of the Ca^{2+} channel. High Ca^{2+} influx or release reduced additional Ca^{2+} influx, thereby preventing proarrhythmic Ca^{2+} overload.

Ca^{2+} must be removed from the sarcoplasm for cardiac muscle relaxation. The relative proportion of Ca^{2+}-processing processes is species-dependent. At the end of the contraction, 70% of the elevated Ca 2+ content of the sarcoplasm is returned to the SR by the SR Ca^{2+} pump. Removal of the remaining 30% into the extracellular fluid is effected by the Na^{+}/Ca^{2+} exchange transporter of the plasma and the Ca^{2+} pump of the plasma.

Calcium-dependent chloride current in myocardial cells

$I_{Cl(Ca)}$ is involved in the repolarization of AP in rabbit, sheep, porcine and canine heart muscle cells. I have already described in the characterization of ionic currents producing action potential that transient outward currents, I_{to1} and I_{to2}, are responsible for phase 1 repolarization in myocardial cells. I_{to2} depolarizes or repolarizes the membrane depending on the instantaneous membrane potential and the Cl^{-} equilibrium potential (E_{Cl}: −50 mV to −30 mV). During the plateau phase of action potential, $I_{Cl(Ca)}$ shifts membrane potential in a negative direction, is present as a repolarizing current, and reduces APD. However, at membrane potentials (at rest) that are more negative than the equilibrium potential of Cl^{-}, $I_{Cl(Ca)}$ has a depolarizing effect and thus may contribute to the formation of EAD and late posterior depolarizations (DAD).

Our group previously found that $I_{Cl(Ca)}$ is activated in canine and human cardiac muscle during phase 1 and during terminal repolarization. In this work, my colleagues also determined that Ca^{2+} entry via the L-type Ca^{2+} channels near the channel is essential for $I_{Cl(Ca)}$ activation, whereas Ca^{2+} released from the sarcoplasmic reticulum only enhances $I_{Cl(Ca)}$ activation.

Calcium-dependent chloride current inhibitor, 9-AC

Several inhibitors of different structure have been used to study Ca^{2+}-dependent Cl^- channels. Previously, our group investigated $I_{Cl(Ca)}$ in isolated canine myocardial cells using two widely used Cl^- channel inhibitors, anthracene-9-carboxylic acid (9-AC) and 4,4′-diisothiocyanostylbene-2,2′-disulfonic acid (DIDS). Then my colleagues proved that the 9-AC-sensitive current is Ca^{2+}-dependent and the Cl^- a charge carrier. Of the two inhibitors, 9-AC is preferred for studying the physiological role of $I_{Cl(Ca)}$ because it did not inhibit the major ion currents that produce AP (I_{Na}, $I_{Ca,L}$, I_{Kr}, I_{Ks}, I_{K1}) at the concentrations tested.

The pro or antiarrhythmic role of calcium-dependent chloride current

In cardiac arrhythmias, the electrical activity of the heart changes. Some of these arrhythmias may be life-threatening tachyarrhythmias such as TdP and ventricular fibrillation. These tachyarrhythmias can cause sudden cardiac death, which is the leading cause of death in Europe and the United States.

Disorders of intracellular Ca^{2+} homeostasis (Ca^{2+} overload and spontaneous release of Ca^{2+} from SR) may also contribute to the formation of TdP. Spontaneous SR Ca^{2+} release activates the cell's Ca^{2+} clearance mechanisms and generates a transient inward current that leads to DAD and triggers triggered activity. The source of the transient inward current may be I_{NCX} in forward mode or $I_{Cl(Ca)}$ activation.

In $I_{Cl(Ca)}$ Ca^{2+} overloaded cells, it can induce DAD and thus be arrhythmogenic. In contrast, the occurrence of EADs and DADs induced under certain conditions increased in canine ventricular cells by inhibiting $I_{Cl(Ca)}$ with 9-AC, indicating the role of $I_{Cl(Ca)}$ in reducing cardiac arrhythmias. Similarly, $I_{Cl(Ca)}$ may play an important role in the prevention of arrhythmias in acidosis in rabbit ventricular myocardial cells. However, this antiarrhythmic function was absent in the EAD model of sheep, where DIDS had little effect. These conflicting results require further studies to determine the pro or antiarrhythmic role of $I_{Cl(Ca)}$.

Proteins responsible for calcium-dependent chloride currents

The molecular identity of $I_{Cl(Ca)}$ is still not fully understood; TMEM16A and Bestrophins are the most likely candidates, the latter at least as Ca^{2+} sensitive channel subunits.

The proteins of the Transmembrane protein 16 (TMEM16) family are anion channels with 8 transmembrane domains, hence the name of the family of proteins, Anoctamin1 or Ano1 for short. More and more information is available on the physiological functions of TMEM16 in smooth muscle as well as in neuronal and other tissues. TMEM16A is expressed in rodent

ventricles and is probably responsible for $I_{Cl(Ca)}$ in ventricular myocytes. Examining TMEM16A, several workgroups recorded ion currents very similar to those of classic $I_{Cl(Ca)}$.

Bestrophin was first described as a gene responsible for Best's vitelliform macular dystrophy. The Cl^- conductance of the retinal pigment epithelial cells of knock-in mice bearing the Best-1 gene mutation was unchanged, but the mutation caused retinal lesions similar to human diseases. All in all, we can assume that Bestrophins are anion channels, but their function in $I_{Cl(Ca)}$ formation is still unclear.

Previously, our group demonstrated the expression of TMEM16A and Bestrophin-3 in both canine and human isolated left ventricular myocytes. Immunocytochemical experiments have also shown that these two proteins and the Cav1.2 channel are colocalized in the sarcolemma, suggesting that Ca^{2+} entering the L-type Ca^{2+} channels directly regulates $I_{Cl(Ca)}$ in canine ventricular myocytes.

Influence of intracellular calcium concentration

In cellular electrophysiological experiments, the intracellular calcium concentration is often buffered by a so-called. calcium chelators, often EGTA and BAPTA. BAPTA has much faster Ca^{2+} binding kinetics, and therefore the two agents are able to buffer Ca^{2+} in different ways in different subcellular compartments.

The so-called sarcolemma and SR in the junctional gap (between the L-type Ca^{2+} channels and the ryanodine receptors), the Ca^{2+} concentration can reach much higher values and kinetically changes faster than the changes in the global Ca^{2+} concentration in the cytoplasm. It is very important to select a calcium chelator with appropriate kinetics for electrophysiological measurements, especially for Ca^{2+} -dependent currents. A wide range of ion currents in the heart is known to be Ca^{2+} -dependent, including Na^+, Ca^{2+}, K^+, Cl^-, Na^+/Ca^{2+} exchange and non-specific cation currents, e.g. TRPM4 current.

Previously, our group investigated the difference between BAPTA and EGTA on $I_{Cl(Ca)}$ and $I_{Ca,L}$. Despite the absence of Ca^{2+} transients in the presence of any buffer, only BAPTA was able to effectively reduce the Ca^{2+} -dependent inactivation of $I_{Ca,L}$, which resulted in greater I_{NISO} (equivalent to L-type calcium current) amplitude and total carried charge and longer action potential. In the presence of BAPTA, $I_{9\text{-}AC}$ (equivalent to the Ca^{2+} -dependent chloride current) was abolished, but EGTA only decreased the current amplitude and the total carried charge.

In voltage-clamp experiments, the buffering of $[Ca^{2+}]_i$ is relatively simple since dialysis of the intracellular compartment by BAPTA Ca^{2+} chelator can be easily performed with a large tip (typically> 2 μm) patch pipette.

Under physiological conditions, action potentials are recorded using conventional pointed microelectrodes, but due to the small diameter of the tip of the measuring electrode, it is not possible to introduce the chelator into the cell via a pipette. In this case, the cell permeable acetoxymethyl ester of Ca^{2+} chelating agent (BAPTA-AM, EGTA-AM) can be used to reduce $[Ca^{2+}]_i$. Such experiments have been conducted for decades, and the results obtained were interpreted solely by $[Ca^{2+}]_i$ buffering despite the fact that BAPTA-AM (and EGTA-AM) interacts with K^+ channels expressed in HEK cells.

TRPM4 current in cardiac muscle cells

28 members of the Tranziens Receptor Potential (TRP) ion channel family are also found in the human body. A member of this huge family is the Tranziens Receptor Potential Melastatin 4 (TRPM4) channel.

The structure of the TRPM4 protein consists of six transmembrane domains. At the N- and C-terminal ends of the protein, several PKA and PKC phosphorylation sites, the ABC motif (ATP-binding casette, ATP-binding cassette), the calmodulin binding site, the Walker B motif (considered as the ATP-binding site), PiP2 (phosphatidyl- inositol-4,5-bisphosphate) binding site and so-called. Contains a coiled coil domain.

TRPM4 is permeable to monovalent cations only, in order of permeability: $Na^+ \sim K^+ > Cs^+ > Li^+$. Under physiological conditions, after activation by increasing intracellular calcium (K_d=400 nM), TRPM4 induces sodium uptake, leading to membrane depolarization. The presence of TRPM4 channels has been described in rat, mouse, bovine and human heart by expression or electrophysiological studies. Gene-dependent mutations in TRPM4 are associated with type I familial heart disease. In addition, genetic alterations in the TRPM4 gene lead to atrial ventricular block, right Tawara stem block, bradycardia, and Brugada syndrome. TRPM4 current also contributes to pacemaker function in rat, mouse, and rabbit, presumably for protection against bradycardia. TRPM4 increases the APD in both atrial muscle and isolated myocardial cells. In rodent ventricular muscles, TRPM4 is also involved in mediating the inotropic effect of β-adrenergic stimulation. Current has been described as being able to reduce angiotensin II-induced hypertrophy.

Because Ca^{2+} -dependent channels do not sort between Na^+ and K^+ ions, they usually generate inward current, which increases the tendency to arrhythmia. In contrast, inhibition of TRPM4 is antiarrhythmic.

TRPM4 Current Inhibitor, 9-phenanthrol

Currently, the pharmacology of TRPM4 is still relatively unknown. In experiments, flufenamic acid or 9-phenanthrol are most commonly used to inhibit current, but flufenamic acid has been shown to be non-selective, and the selectivity of TRPM4 for 9-phenanthrol in the heart has not been proven to date. It is important to note that the cardiac electrophysiological effects of 9-phenanthrol have so far been studied only in mice, a species whose action potential and the ion currents that produce it are very different from those of humans.

Aims

We aimed to investigate the role of $I_{Cl(Ca)}$ in the formation of spatial and temporal heterogeneity of left ventricular repolarization. We determined this by using two methods that reflect physiological conditions (physiological intra- and extracellular ion composition, retained intracellular Ca^{2+} homeostasis, AP voltage command). On the one hand, we wanted to use conventional sharp microelectrode measurements and on the other hand, the action potential voltage clamp (APVC) technique to characterize $I_{Cl(Ca)}$ as a 9-AC sensitive current in cells from different regions of the left ventricular myocardium. Furthermore, normalized expression of TMEM16A and Bestrophin-3 proteins in isolated cardiac cells was determined by Western blotting.

Earlier results from our study showed that the effect of calcium chelator on BAPTA-AM and I_{Ks} and I_{Kr} potassium channel blockers, respectively, influenced BAPTA-AM action potential morphology by the potassium channel blocker selectivity. Both BAPTA-AM and EGTA-AM interact with K^+ channels expressed in HEK cells. It is also known that drug-channel interactions can be significantly different for native and expressed ion channels. Therefore, we aimed to investigate whether BAPTA-AM affects the action potential and I_{Kr} current of left ventricular myocytes.

We wanted to investigate the role of the TRPM4 current in the formation of action potential morphology and the behavior of the current during AP. To this end, we intended to use the current-specific inhibitor, 9-phenanthrol, in action-potential clamp measurements on left ventricular myocardial cells. As this method requires as much selective ion channel blocking agent as possible, we first designed experiments to test the selectivity of 9-phenanthrol.

Canine ventricular cells were selected for all of our studies because the electrophysiological properties of this preparation are most similar to those of the human myocardium.

Materials and Methods

Isolation of canine heart muscle cells

Electrophysiological and gene expression experiments were performed on myocardial cells isolated enzymatically from the left ventricle of the canine heart. Cells were obtained from hearts of mixed-sex, mature dogs cultured for experimental purposes using anterograde segment perfusion techniques. The experiments carried out were in line with the principles of the 'Guide for the Care and Use of Laboratory Animals' and the 'Guide to the Care and Use of Experimental Animals' and the Helsinki Declaration. The experimental protocol was also approved by the Workplace Animal Ethics Committee of the University of Debrecen.

Myocardial cells were isolated by segment perfusion and collagenase digestion. Due to the nature of cell isolation, most of our measurements were performed on midmyocardial (MID) cells. However, for some of our experiments, cardiomyocytes isolated from the subepicardial (EPI) and subendocardial (ENDO) regions of the left ventricle were used. For a separate group of experiments, cells were collected from the apical and basal portions of the MID layer of the left ventricle wall. In the final cell suspension, the cells are regular rectangular, sharp-edged, with a clear, intact transverse stripe of cytoplasm.

Electrophysiology

2-3 drops of cell suspension were placed in a 1 mL volumetric plexiglas and continuously perfused with Tyrode solution provided by a gravity driven system. In our experiments, a constant temperature of 37 °C was set with a temperature controller. Cells were visualized using an inverted microscope on an anti-vibration table in a Faraday cage. Cells were punctured with a micropipette or pointed microelectrode to record subsequent AP or ion current. They were prepared using a capillary puller. Microelectrodes and micropipettes were contacted with cells using a mechanical macromanipulator and a three-way piezoelectric micromanipulator. Electrical analog signals from the cells were routed and amplified via a headstage. The amplified analog signals were also monitored on an oscilloscope. For computer control and data acquisition, pClamp 10 software was used. The connection between the amplifier and the computer software was made possible by a digitizer. We used Microsoft Excel software to evaluate the raw data, and Origin and PowerPoint graphics software were used to generate the data from the evaluation.

Measurement of action potentials by pointed microelectrode technique

Membrane potential measurements were performed using a high-resistance glass microelectrode technique developed for a single cell system. A high resistance (25−40 MΩ) microelectrode filled with 3 M KCl was used to drive the APs. The amplifier was used in current-clamp mode and the cells were stimulated with a pulse generator. The APs were evaluated using a Microsoft Excel macro programmed by our workgroup. The parameters calculated by the software are: resting membrane potential (RMP), maximum depolarization rate (V^{+}_{max}), AP amplitude (APA), phase 1 repolarization amplitude (voltage difference between AP peak and pre-dome notch), and slope, duration of AP 90% repolarization (APD_{90}), half of APD_{90} membrane potential ($plateau_{50}$), and terminal repolarization slope (V^{-}_{max}).

Ion currents measurement by voltage clamp technique

Ion currents were measured in the full-cell configuration of the patch-clamp technique using an amplifier in a voltage-clamp mode. Micropipettes (2-3 MΩ) were used for the measurements. In a full-cell arrangement of the patch-clamp technique, the internal solution of the micropipette communicates with the intracellular space of the cells. By changing the composition of the internal solution we were able to influence the free Ca2 + concentration of the cells with the help of calcium chelator. The action-voltage voltage-clamp technique is the most suitable method for studying ion currents involved in the formation of action potentials under physiological conditions. In such measurements, the cell's own action potential, recorded in a current-clamp mode, was used as a command signal on the same cell.

Protein sample preparation and Western blot analysis

Enzymatically isolated cardiomyocytes from various transmural regions of the left ventricle (EPI, ENDO, MID) and the apical and basal regions of the left ventricle midmiocardial layer were used for molecular biological studies. The optical densities of the TMEM16A and Bestrophin 3-specific bands were normalized to the optical density of the β-actin-specific bands of the samples.

Statistical analysis

Data reported are arithmetic mean of experimental results ± standard error of the mean. When comparing the groups, one-way analysis of variance, Student's two-sample t-test or self-controlled t-test was used according to the statistical questions. Differences between data were considered significant at $p < 0.05$.

Results

The contribution of $I_{Cl(Ca)}$ to the AP contour differs in various transmural layers of canine left ventricular myocardium

First the effect of $I_{Cl(Ca)}$ inhibition achieved by 0.5 mmol/L 9-AC was evaluated in isolated myocytes obtained from various layers of the left ventricular anterior wall. After the stabilization of the AP shape, APs were recorded in steady-state conditions starting with 5 s cycle length (CL), and then procedure was repeated using progressively smaller CLs in control solution. After recording steady-state APs at five different CLs the CL was changed back to 5 s and 9-AC was perfused. When the effect of 9-AC developed (typically 7–8 min) the AP recordings at changing CLs were repeated as before. Finally the washout of the inhibitor was per- formed to test the reversibility of 9-AC-induced AP changes.

9-AC reduced phase-1 repolarization of AP in all cell types studied. $I_{Cl(Ca)}$ inhibition increased APD_{90} in MID and ENDO cells but did not significantly change APD_{90} values of EPI cells. Action potential duration was altered by 9-AC in a reverse rate-dependent manner in MID and ENDO cells. Every 9-AC-induced change of AP parameters was reversible within the 10–15 min of washout. Resting membrane potential (RMP) and V_{max} values were not altered by 9-AC.

To study the possible reasons of the observed transmural differences of 9-AC-induced AP changes, 1 mmol/L 4-aminopyridine (4-AP, inhibitor of I_{to1}) was applied in EPI cells to reduce phase-1. This intervention altered the AP contour of EPI cells in a way that it became similar to APs of ENDO cells. The application of 9-AC in the continuous presence of 4-AP altered AP parameters the same way as it was observed in ENDO cells namely 9-AC prolonged APD in 4-AP pretreated EPI cells.

Apico-basal differences in 9-AC-induced changes of AP parameters

Similar experiments were performed on cells isolated from the apical and basal parts of the left ventricular midmyocardium. APD_{90} was increased while phase-1 repolarization was reduced by 9-AC similarly in both types of cells. The reverse rate-dependent lengthening of AP was greater in basal cells and this difference was significant at 0.5 and 1 s CLs. In concordance with our results with cells isolated from various transmural layers, 9-AC had no influence on RMP and V_{max} values and its effects on AP parameters were reversible.

Equal $I_{Cl(Ca)}$ densities, TMEM16A and Bestrophin-3 expression levels in cardiomyocytes obtained from various left ventricular regions

To examine the possible reason for the previously described differences in 9-AC-induced AP changes we looked for any potential differences in $I_{Cl(Ca)}$ densities and protein expression levels of TMEM16A and Bestrophin-3, the most likely candidates responsible for $I_{Cl(Ca)}$. 9-AC-sensitive current densities were measured using conventional square pulses in cells of various origin of the left ventricle. $I_{Cl(Ca)}$ densities were equal regardless of the origin of the studied cell. This was the case not only for the transmural but also for the apico-basal gradient. $I_{Cl(Ca)}$ started to activate at −20 mV and had its highest value at +60 mV in all studied cell types similarly to our earlier studies.

The expression levels of both TMEM16A and Bestrophin-3 were detected together with that of β-actin. Similarly to current densities, equal expression levels were observed for both TMEM16A and Bestrophin-3 when normalized to β-actin expression in all studied cell types.

Based on the square pulse voltage clamp data and protein expression results, the previously seen differential 9-AC-induced AP changes could not due to the differences in channel expression.

Transmural heterogeneity of $I_{Cl(Ca)}$ profiles measured with APVC

To explain the cause of differences in 9-AC-induced AP changes among left ventricular cells of various origins we studied $I_{Cl(Ca)}$ profiles, recorded as $I_{9\text{-}AC}$, with APVC. Using this technique we could activate $I_{Cl(Ca)}$ with the previously recorded own AP of each studied cell as voltage command to test the importance of AP contour in the activation of the current. Moreover, the pipette solution did not contain any calcium buffers or Ca^{2+} so $[Ca^{2+}]_i$ was not altered artificially. $I_{9\text{-}AC}$ profiles were similar in dynamics in each cell type derived from various transmural regions of the left ventricular wall regardless of the marked and well- known difference in the shape of the APs. $I_{9\text{-}AC}$ profiles consisted from an early, large, and fast outward component corresponding to phase-1 of APs followed by a late, small, and inward component. However, both the peak current density and the normalized total charge values of the outward component were statistically larger in EPI cells compared to ENDO ones.

Dependence of $I_{Cl(Ca)}$ profiles on the shape of the AP

To further investigate the possible reason for the greater current peak and total charge values measured in EPI cells with APVC and the markedly different actions of 9-AC on APs of EPI cells the following strategy was used. Typical ENDO and EPI APs were selected and used as voltage commands in APVC experiments in both ENDO and EPI cells. The theory

behind this experiment was to test the importance of the shape of the AP command waveform in activation of $I_{Cl(Ca)}$. $I_{9\text{-}AC}$ profiles were very similar in dynamics with both stimulus waveforms in both ENDO cells and EPI cells. There was however a difference in the peak current density and total charge values of the early outward component as activating $I_{Cl(Ca)}$ with EPI waveforms always resulted in larger values independent from which cell type was used to record $I_{Cl(Ca)}$.

Role of $I_{Cl(Ca)}$ in EAD generation and short-term variability of repolarization (SV)

EAD is a depolarization occurring before complete repolarization, during the plateau phase of the AP and considered to be responsible for the initiation of malignant cardiac arrhythmias. During our experiments 0.5 mmol/L 9-AC induced EADs in a small percentage of cells only at long CL stimulation. Increasing $[Ca^{2+}]_i$ by the application of the non-specific β-adrenergic agonist isoproterenol (ISO, 10 nmol/L) did not evoke EADs at any studied CLs in the absence of 9-AC but the simultaneous application of the two compounds result- ed in much higher EAD occurrence, especially at long CLs.

The *short-term variability* of QT interval has a better predictive value regarding the development of cardiac arrhythmias than the *duration* of the QT interval itself. As QT interval corresponds to the duration of ventricular AP, short-term variability of QT interval can be represented as short-term variability of AP duration (SV) on the cellular level. It must be emphasized that these experiments were done at 1 Hz steady-state pacing, therefore the recorded changes in either APD or SV could not be due to EAD occurrence. To better illustrate SV, Poincaré diagrams are usually plotted where either the duration of APs (APD_{90}) or QT intervals are presented as a function of the previous APD_{90} or QT interval duration values. 9-AC increased APD_{90} whereas the addition of ISO abolished this AP prolongation. Average of SV values in the 24 studied cells was larger in the presence of 9-AC (3.1 ± 0.2 ms) but the addition of ISO reduced the SV to a smaller value (1.8 ± 0.2 ms) than that calculated in control condition (2.3 ± 0.1 ms). However, without 9-AC pretreatment ISO decreased SV to an even smaller value (1.4 ± 0.1 ms). Even more importantly, upon evaluating the dispersion of differences in consecutive APD_{90} values in the presence of 9-AC the cumulative distribution curve was shifted toward greater beat-to-beat variability, indicating the increase of differences in consecutive APD_{90} values.

Effect of BAPTA-AM on action potential morphology

The most conventional way to study the effect of changes in $[Ca^{2+}]_i$ on action potential configuration is loading the cell interior with the Ca^{2+} chelator BAPTA using its cell-permeant

acetoxy-methylester form, or alternatively, to load the cells with Ca^{2+} using the Ca^{2+}-ionophore A23187. Exposure to 5 μM BAPTA-AM lengthened, while to A23187 shortened the duration of action potentials measured at 90% level of repolarization (APD_{90}). These changes developed gradually in a time-dependent manner although in the case of BAPTA-AM there was an initial rapid rise in APD_{90}. Actions of BAPTA-AM and A23187 failed to fully saturate within the recording period of 30 min, probably due to the progressive intracellular accumulation of free BAPTA resulting in a more and more effective buffering of $[Ca^{2+}]_i$ in the first case and accumulation of Ca^{2+} in the second one. Furthermore, the changes in APD_{90} were accompanied with characteristic changes in action potential morphology, since reduction of $[Ca^{2+}]_i$ shifted the plateau potential to more positive voltages, while its elevation resulted in a marked depression of the plateau.

The effect of BAPTA-AM was studied under conditions when the density of $I_{Ca,L}$ was manipulated. In the presence of the Ca^{2+}-channel blocker nisoldipine the BAPTA-AM-induced APD-lengthening was negligible (although statistically significant) and only transient since it disappeared after 20 min exposure to BAPTA-AM. In contrast, increasing $I_{Ca,L}$-density with BAY K8644 markedly augmented the BAPTA-AM-induced prolongation of APD_{90}. It is worthy of note that BAPTA-AM showed a biphasic effect in the presence of BAY K8644. After 5 min exposure to BAPTA-AM APD_{90} reached its maximum, and after this it shortened gradually reaching a local minimum around 15 min of BAPTA-AM treatment.

The effects of nisoldipine and BAY K8644 on action potential configuration (plateau-depression by nisoldipine and elevation by BAY K8644) were qualitatively similar to those seen with A23189 and BAPTA-AM, respectively. Taken these findings together, one might logically conclude that the reduction of $[Ca^{2+}]_i$ lengthens while its elevation shortens the action potential, furthermore, these effects are likely mediated *via* the Ca^{2+}-dependent inactivation of $I_{Ca,L}$.

Contrasting to the argumentation above, when the cells were pretreated with I_{Kr} blocker (100 nM dofetilide or 300 nM E 4031) BAPTA-AM shortened APD_{90} instead of lengthening it. The applied concentrations of I_{Kr} blockers eliminated more than 75% of I_{Kr}. This shortening effect, however, was temporary - strongly resembling the transient APD_{90}-shortening tendency observed with BAPTA-AM in the presence of BAY K8644 following the immediate APD_{90} lengthening effect of the former. On the other hand, the APD_{90}-lengthening effect of 100 nM dofetilide was significantly reduced in the presence of 5 μM BAPTA-AM. The effect of BAPTA-AM on action potential morphology was sensitive exclusively to the density of I_{Kr}, since blocking I_{Ks} with 1 μM HMR 1556 failed to alter the effect of BAPTA-AM on action potential duration. The effects of 5 min pretreatment with nisoldipine, BAY K8644, dofetilide

and E 4031 on APD_{90} were comparable in magnitude to the those observed after longer exposures, lasting for 20 - 30 min. This is important because any change in APD_{90} recorded in the presence of BAPTA-AM cannot be attributed to time-dependent progression of the effect of the drug used for pretreatment.

To demonstrate that not BAPTA - but only BAPTA-AM - is responsible for the observed alterations in action potential morphology, myocytes were superfused with 5 μM BAPTA. No change in action potential duration or in other parameters were observed after 30 min exposure to BAPTA indicating that the presence of the acetoxy-methyl group in the molecule is essential for the development of acute APD-lengthening effect of BAPTA-AM. Both the duration as well as the configuration of action potentials failed to alter significantly in untreated canine ventricular cells within the 60 min period of recording.

Effect of BAPTA-AM on I_{Kr} current

In the first set of experiments, the effect of externally applied BAPTA-AM was studied on I_{Kr} in 5 ventricular myocytes using the conventional patch clamp technique. The density of I_{Kr} was evaluated by the amplitude of I_{Kr} tail currents recorded upon repolarization to –40 mV. Exposure of the cells to 5 μM BAPTA-AM for 5 min decreased the density of I_{Kr} to 32 ± 8 % of the control (from 0.39 ± 0.05 to 0.12 ± 0.03 A/F, $P < 0.05$, $n = 5$) in a partially reversible manner, since the current amplitude returned to 76 ± 5% of its control value during a 5 min period of washout with BAPTA-AM-free superfusate. As the pipette solution contained 10 mM BAPTA in these experiments, suppression of I_{Kr} by BAPTA-AM was independent of intracellular Ca^{2+} buffering.

Conventional voltage clamp experiments are not ideal to visualize the consequences of an ion current blockade during the action potential. Therefore action potential voltage clamp experiments have been designed to monitor the changes in I_{Kr} current profile following superfusion with 5 μM BAPTA-AM. Since this technique is based on pharmacological extraction of I_{Kr} (using 1 μM E 4031), measurements had to be performed in two populations of myocytes. The density of I_{Kr} measured in the absence (control) and presence of external BAPTA-AM was 0.50 ± 0.03 and 0.17 ± 0.06 A/F ($n = 7$ and $n = 5$, respectively, $P < 0.05$), corresponding to a reduction of 66%. There was a similar difference in the amount of charge carried by I_{Kr} during the action potential: 22 ± 5 versus 6 ± 2 mC/F ($n = 7$ and $n = 5$, respectively, $P < 0.05$), a reduction to 27% of the control value. The Ca^{2+}-buffering effect of BAPTA-AM could be excluded in these experiments as well, because the cells had been pre-loaded with BAPTA before their exposure to external BAPTA-AM.

Effects of 9-phenanthrol on the major cardiac ionic currents

Concentration-dependent effects of 9-phenanthrol on ionic currents were studied at 1, 3, 10, and 30 μM concentrations under conventional voltage-clamp. Each concentration was applied for 5 min; the washout at the end of the experiment lasted for 10 min. The inward rectifier K^+ current (I_{K1}) was recorded with hyperpolarizing pulses from –80 mV to –130 mV applied for 200 ms at 0.05 Hz stimulation rate; the current was determined at the end of the pulse. I_{K1} was significantly decreased by 10 μM or higher concentrations of 9-phenanthrol in a partially reversible manner.

The rapid delayed rectifier K^+ current (I_{Kr}) was activated by 0.5 s depolarizing pulses to 40 mV arising from the holding voltage of –40 mV at 0.05 Hz stimulation rate. I_{Kr} was assessed as tail currents recorded following repolarization to the holding voltage. L-type calcium current ($I_{Ca,L}$) and I_{Ks} were pharmacologically suppressed. The amplitudes of the I_{Kr} tail currents were progressively decreased by increasing concentrations of 9-phenanthrol, which effect was only partially reversible. $I_{Ca,L}$ was recorded at 10 mV during 200 ms depolarizations arising from the holding voltage of –80 mV at 1 Hz stimulation rate. The test pulse was preceded by a short (20 ms) depolarization to –40 mV to eliminate Na^+ current. At 10 mV in the presence of increasing concentrations of 9-phenanthrol, a progressively increasing steady outward current could be detected, which was only partially reversible. $I_{Ca,L}$, measured as the difference between the peak and the steady component at the end of the depolarization pulse, was not affected by 9-phenanthrol up to the concentration of 30 μM.

The transient outward potassium current (I_{to1}) was activated by depolarization to 40 mV from the holding voltage of –80 mV at 0.05 Hz stimulation rate. Before clamping to the test voltage, a prepulse to –40 mV was applied for 10 ms to activate then inactivate fast Na^+ current, while $I_{Ca,L}$ and I_{Ks} were suppressed by 1 μM nisoldipine and 1 μM HMR 1556, respectively. Similarly to what could be observed with I_{Ca} measurements, a steady outward current developed in the presence of 9-phenanthrol. I_{to1} was measured as the difference between the peak and the steady component right after full inactivation (40 ms from the start of the 40 mV depolarization). I_{to1} was significantly inhibited by 10 and 30 μM 9-phenanthrol in a largely reversible manner.

As mentioned earlier, an outward current could be detected in the presence of 9-phenanthrol at voltages of 10 mV and 40 mV. To visualize this current, 200 ms voltage ramps, going from 5 to –120 mV, were applied at 1 Hz stimulation rate first in the absence, then in the presence of 9-phenanthrol. The current–voltage relation obtained using the ramp deviated at its negative

branch (likely due to 9-phenanthrol-induced inhibition of I_{K1}) and also in the voltage range above –40 mV. To check whether this current is also present under steady-state conditions, the 9-phenanthrol-induced extra current was measured at steady membrane voltages of –40, 10, and 40 mV. Indeed, the extra outward current was evident from 3 μM 9-phenanthrol at 10 and 40 mV, its amplitude increased with the concentration of 9-phenanthrol applied, and importantly, the current was not present at –40 mV.

Effects of 9-phenanthrol on action potential morphology

In contrast to the voltage-clamp experiments, where 10 mM BAPTA was present internally to strongly buffer cytosolic Ca^{2+} and therefore to prevent the Ca^{2+}-dependent activation of TRPM4, action potentials were recorded under conditions of normal Ca^{2+} cycling using KCl-filled conventional microelectrodes. Sixteen cells were exposed to cumulatively increasing concentrations of 9-phenanthrol. Out of these cells, 6 were treated with 9-phenanthrol concentrations of 1, 3, 10, and 30 μM, while 12 cells were exposed only to 10 and 30 μM concentrations. Each concentration was superfused for 5 min and a 10-min period of washout was applied for 10 min at the end of the experiment.

9-Phenanthrol caused a concentration-dependent depression of the plateau potential measured at 50% of APD ($plateau_{50}$). This effect was significant from 3 μM and was reversible upon washout. The maximal rate of depolarization (V^{+}_{max}), which is considered as an approximate measure of fast Na^{+} current density, was also decreased significantly from the concentration of 3 μM, but it was only partially reversible upon washout. Action potential amplitude (from 107.5 ± 1.6 to 99.7 ± 2.1 mV, $n = 16$, $p < 0.05$) and the overshoot potential (from 25.3 ± 1.8 to 19.2 ± 2.3 mV, $n = 16$, $p < 0.05$) were also significantly decreased by 30 μM 9-phenanthrol, without significantly changing the resting membrane potential (–82.2 ± 0.9 versus –80.5 ± 1.2 mV in the absence and presence of 9-phenanthrol, respectively, $n = 16$, not significant).

The slope of early repolarization (phase 1), which may reflect I_{to1} density was significantly reduced by 9-phenanthrol. This was the strongest effect of the drug on action potential configuration: it was significant from 3 μM concentration and was fully reversible. Similarly, the amplitude of phase 1 repolarization, measured between the overshoot potential and the notch of the incisura, was also reduced significantly by 30 μM 9-phenanthrol (from 22.5 ± 2.0 to 6.0 ± 1.7 mV, $n = 16$, $p < 0.05$). The rate of terminal repolarization (V^{-}_{max}), which correlates well with the density of I_{K1}, was reduced significantly by 10 and 30 μM 9-

phenanthrol, however, this effect was less pronounced as that on phase 1 and was only partially reverted by washout.

The effect of 9-phenanthrol on APD measured at 90% level of repolarization (APD_{90}) was not uniform in the studied myocytes. The average values corresponding to the total 16 cells studied showed no significant change up to 10 μM concentration, but APD_{90} began to increase in the presence of 30 μM which effect progressively continued during the washout. Similar response was seen in 12 out of 16 myocytes studied. However, 4 myocytes responded with significant shortening of APD_{90}. Upon washout, however, APD_{90} increased above the control level in these cells. Other studied action potential parameters were similarly affected by 9-phenanthrol in the 2 groups of myocytes. Regarding the baseline action potential parameters, measured in control conditions before the application of 9-phenanthrol, the following parameters were statistically significant between the 2 groups of cells: the voltages of both $plateau_{50}$ and at the most negative point at the end of phase 1 were higher; while APD_{90} was smaller in cells responding with action potential prolongation compared with the cells responding with action potential shortening to 9-phenanthrol treatment.

Discussion

Role of calcium-dependent chloride current in spatial and temporal heterogeneity of repolarization

Influence of $I_{Cl(Ca)}$ on AP configuration and spatial heterogeneity of repolarization

Similarly to our previous study 9-AC did not modify V_{max} in any studied cell type indicating its lack of action on fast Na^+ current. This makes the drug suitable for studying the role of $I_{Cl(Ca)}$ even during AP measurements. 9-AC prolonged APD in both ENDO and MID cells in line with previous findings. In EPI cells, however, there was no AP prolongation. This can be due to a possible shortening effect induced by I_{Kr} accumulation and also by a smaller Ca^{2+} influx in the absence of a pronounced spike and dome AP configuration. This AP shortening seems to overcome the AP prolongation likely induced by 9-AC. Phase-1 repolarization was reduced by 9-AC in all studied cell types. Upon the reduction of phase-1 (by partial blockade of I_{to1} achieved by 1 mmol/L 4-AP) the shape of the AP in EPI cells became similar to those of ENDO ones. When 9-AC was applied in this condition, changes of AP parameters became practically identical to those observed in ENDO cells. These results suggest that the lack of 9-AC action on APD of EPI cells were due to the pronounced spike and dome configuration of AP created by the large I_{to1} density in these cells. This is in good agreement with the conclusion of Zygmunt et al. who suggested an important role for I_{to1} in the activation of subsequent ion currents like $I_{Cl(Ca)}$ and $I_{Ca,L}$.

Our results are in good agreement with an earlier study despite the differences in the experimental conditions. Verkerk et al. used DIDS to inhibit $I_{Cl(Ca)}$ in rabbit left ventricular myocytes measured with patch-clamp configuration while here 9-AC was applied during sharp microelectrode recording in canine left ventricular myocytes. Still, they found no AP prolongation in EPI cells except for the fastest stimulation rate of 3.33 Hz. Moreover, similarly to our observation, blockade of $I_{Cl(Ca)}$ prolonged the AP in EPI cells at 1 Hz only when DIDS was applied in the presence of I_{to1} inhibition. A canine study reported similar findings to our results as APD was increased when phase-1 repolarization was slow and shallow (like in MID and ENDO cells).

Blockade of $I_{Cl(Ca)}$ prolonged APD of MID, ENDO and also 4-AP pretreated EPI cells in a reverse rate-dependent manner. This concurs with our previous findings about rate-dependent changes of APD in canine and in other mammalian species. Moreover, two other factors might contribute to the 9-AC-induced, rate-dependent AP prolongation. One is the

rate-dependent variation of $[Ca^{2+}]_i$ as it was shown that $[Ca^{2+}]_i$ is larger upon the increase of stimulation rate both during steady-state as well as without steady-state. Moreover, $[Ca^{2+}]_i$ correlates well with $I_{Cl(Ca)}$ measured as $I_{9\text{-}AC}$ with square pulses. Another factor is the rate-dependent behavior of I_{to1} density as the availability of its channels reduces at fast stimulation rates due to the relatively slow recovery from inactivation. This is reflected in the fact that phase-1 showed rate-dependent properties in control conditions as it was larger at long CLs. Moreover, this rate-dependence was also present when $I_{Cl(Ca)}$ was blocked by 9-AC in all five studied cell types (not shown). Therefore, the rate-dependence of phase-1 seems to be rather modulated by the recovery from inactivation kinetics of I_{to1} than the magnitude of $I_{Cl(Ca)}$. This emphasizes that reverse rate dependency is an intrinsic property of the myocardium. Therefore, similarly to the study performed on rabbit cells the role of $I_{Cl(Ca)}$ in the reduction of transmural APD heterogeneity is more pronounced at slow heart rates. This makes the contribution of $I_{Cl(Ca)}$ even more important as the heterogeneity of repolarization is the greatest at slow stimulation rates.

Our study is the first to describe the apico-basal contribution of $I_{Cl(Ca)}$ to AP configuration. Our baseline AP parameters were similar to those published earlier and 9-AC-induced changes of AP parameters in the apico-basal direction were similar to those observed in the transmural direction. Again, the heterogeneity of AP repolarization was reduced by $I_{Cl(Ca)}$ and this action was the strongest at slow stimulation rates.

Transmural difference in $I_{Cl(Ca)}$ and its underlying ion channel proteins

$I_{9\text{-}AC}$ could be used to explore the contribution of $I_{Cl(Ca)}$ to the AP in closely physiological conditions (without the application of intracellular Ca^{2+} buffering during APVC). Regional differences, however, were not studied previously with APVC despite some earlier studies where either a "typical" AP was applied as the command signal during perforated patch recording using nonspecific blockers to dissect $I_{Cl(Ca)}$ or 10 mmol/L EGTA was applied in the pipette solution interfering with physiological Ca^{2+} homeostasis. The current density of $I_{9\text{-}AC}$ early outward component was statistically larger in EPI cells compared to what was recorded in ENDO ones. This EPI-ENDO difference was unexpected as a previous study did not report any transmural difference in $I_{Cl(Ca)}$ density in the canine left ventricle. It must be noted that the result of that study is in full agreement with our results as $I_{Cl(Ca)}$ densities of EPI and MID cells were compared. $I_{Cl(Ca)}$ was not studied in ENDO cells and conventional square pulses were used with the application of the nonspecific blocker (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) of the current. Similarly, although 9-AC was used

both during APVC and conventional voltage-clamp measurements, in the latter case no difference was found in $I_{Cl(Ca)}$ densities of cells obtained from various transmural layers just as upon comparing midmyocardial cells from apical or basal origin. In line with this, the densities of TMEM16A and Bestrophin-3 proteins (two likely candidates for mediating $I_{Cl(Ca)}$) both normalized to β-actin were equal regardless of the origin of the samples. In contrast to present results, the amplitude of $I_{Cl(Ca)}$ measured with square wave voltage clamp was significantly greater in feline epicardial cells compared to endocardial ones suggesting a potential interspecies difference.

Larger $I_{Cl(Ca)}$ density of EPI cells is determined by the contour of AP

The current density of $I_{9\text{-}AC}$ early outward component was statistically larger in EPI cells than that of ENDO ones in APVC conditions and 9-AC induced AP prolongation in ENDO but not in EPI cells. On the contrary, $I_{9\text{-}AC}$ density was equal with square pulse voltage-clamp measurements. It must be noted that during square pulse measurements the stimulus waveform is quite different from the physiological one. Under APVC, however, the previously recorded, own AP was used for physiological stimulation on each studied cell. To test the hypothesis that the AP waveform could be responsible for the larger $I_{Cl(Ca)}$ density of EPI cells we used two previously recorded characteristic ENDO and EPI AP waveforms. ENDO AP possessed only a slow and small phase-1 whereas EPI AP had a rapid and large phase-1 followed by a pronounced dome. Observations were consistent with our hypothesis as the characteristic EPI AP waveform induced a larger early outward $I_{Cl(Ca)}$ current than the ENDO AP waveform no matter whether the waveforms were applied on EPI or ENDO cells. These results can be explained based on what our group has previously found in case of the nisoldipine-sensitive current on canine and human left ventricular myocytes. It was shown that the charge carried by the nisoldipine-sensitive current was significantly larger during EPI AP compared to ENDO one. This could result in a greater activation of $I_{Cl(Ca)}$ during the early phase of the AP. It is hard to predict the exact mechanism as the membrane potential also influences the activation of $I_{Cl(Ca)}$ beside the $[Ca^{2+}]_{cleft}$. Although the existence of a marked phase-1 during the EPI AP favors the entry of Ca^{2+} due to its increased driving force and the I-V characteristics of the LTCCs but this more negative membrane potential actually acts against Cl^- influx. Nevertheless, it seems that the overall action of a more pronounced phase-1 (EPI AP) is the increase of the early outward component of $I_{Cl(Ca)}$. This larger current is definitely not due to the increased density of channel proteins in EPI cells as the expression of TMEM16A and Bestrophin-3 was equal in both transmural and apico-basal

direction. Although the expression level of $Ca_v1.2$, the pore-forming subunit of LTCCs was not studied in ENDO cells but it was equal in EPI and MID cells, similarly to the equal density of TMEM16A and Bestrophin-3 described in the present study.

Influence of $I_{Cl(Ca)}$ on temporal variability of cardiac repolarization and EAD formation

Prolongation of repolarization is considered as a risk factor of cardiac arrhythmias as it can lead to EADs and TdP. The short-term variability of repolarization is considered to be even more important as a predictor of cardiac arrhythmias highlighted by a position statement and consensus guidance were endorsed by the European Heart Rhythm Association jointly with the ESC Working Group on Cardiac Cellular Electrophysiology recently. According to the previously mentioned studies, any SV reducing intervention could have an antiarrhythmic property. It must be emphasized that all of our experiments where SV was calculated were done at 1 Hz steady-state pacing, therefore the recorded changes in either APD or SV could not be due to EAD occurrence. The application of 9-AC induced an increase in SV suggesting an antiarrhythmic role for $I_{Cl(Ca)}$. This antiarrhythmic property of $I_{Cl(Ca)}$ is further supported by the fact that EADs were detected upon the blockade of $I_{Cl(Ca)}$. Another evidence supporting the antiarrhythmic role of $I_{Cl(Ca)}$ is the even larger EAD incidence in the simultaneous presence of 9-AC and ISO. ISO is well known to increase $[Ca^{2+}]_i$ and therefore most probably $[Ca^{2+}]_{cleft}$ as well. This leads to a larger $I_{Cl(Ca)}$ which upon its blockade by 9-AC leads to a greater EAD incidence.

The value of SV calculated with the formula used in this study and also in earlier publications is not necessarily the most sensitive way to indicate if the APD of only a few beats greatly differ from the average. This is especially the case when the APD of 50 consecutive APs are used. However, a large, sudden change in consecutive APD values occurring in a non-uniform manner in cells of various origins within the myocardium will more effectively trigger an arrhythmic event than a gradual and simultaneous increase in APD during the development of the action of a drug. Therefore the difference between consecutive APD values were grouped in ms ranges and the overall probability of their appearance was calculated in order to detect any unusually short or long AP among the 50 analyzed one more easily. Using this analysis, 9-AC shifted the curve to the right, indicating an increase of differences between consecutive APD values, hence an arrhythmogenic effect. Moreover, the protective effect of ISO was smaller in the presence of 9-AC as the leftward shift of the curve by ISO was greater in the absence than in the presence of 9-AC. Similarly, in the presence of ISO the value of SV was smaller when 9-AC was not applied. Therefore it seems that $I_{Cl(Ca)}$ contributes to the ISO

induced reduction of SV. These results all support a protective role of $I_{Cl(Ca)}$ against cardiac arrhythmia together with the previously mentioned spatial reduction of APD heterogeneity.

Our previous study is congruent with the current results as a protective role of $I_{Cl(Ca)}$ was suggested in cesium and ouabain induced canine ventricular myocyte EAD model as well. In contrast, on sheep ventricular cells DIDS barely had any action on parameters of EADs. Apart from the different species, the ovine study used norepinephrine to provoke EADs and studied the action of DIDS, a non-specific blocker of $I_{Cl(Ca)}$ which also inhibits Na^+ channels on various parameters of EADs. In the current study, however, EADs were not provoked but their incidence was studied in the absence or presence of 9-AC, a more specific blocker of $I_{Cl(Ca)}$. Due to these differences it is rather hard to compare these studies.

It must be mentioned that DAD formation can also provoke cardiac arrhythmias by initiating TdP. DADs can be produced not only by the forward mode I_{NCX} but also by $I_{Cl(Ca)}$ at least in some species and cell types. Indeed, it was shown that activation of $I_{Cl(Ca)}$ can be responsible for DAD generation in Ca^{2+}-overloaded canine ventricular, rabbit atrial, ventricular and Purkinje and ovine Purkinje and ventricular cells. The presence of ISO-induced chloride current (and DADs) was not detected in canine ventricular cells but in the same experimental conditions a membrane depolarization was described in isolated guinea pig ventricular myocytes. It might be that the application of 1 μmol/L ISO for 20 s was not enough to induce Ca^{2+} overload in canine cells, hence activation of $I_{Cl(Ca)}$ and DADs in that study. Similarly, we did not detect any DADs during our experiments even in the presence of ISO, indicating that 10 nmol/L ISO does not induce Ca^{2+} overload in canine ventricular myocytes. It was possible to generate DADs by overloading canine cells with Ca^{2+} using ISO, but it had to be applied in 100 times higher dose together with ouabain.

Summary and potential relevance

The major findings of the present study suggest a protective role for $I_{Cl(Ca)}$ against risk of arrhythmias in the canine left ventricular myocardium. $I_{Cl(Ca)}$ achieves this by the reduction of both spatial and temporal heterogeneity of repolarization. As it was suggested earlier inhibition of $I_{Cl(Ca)}$ could be useful to reduce the Ca^{2+} overload induced DADs. At the same time, however, the blockade of $I_{Cl(Ca)}$ might increase the risk of cardiac arrhythmias by increasing spatial and temporal heterogeneity of repolarization and EAD formation. As it was shown the incidence of EAD formation was even higher when the blockade of $I_{Cl(Ca)}$ was established in the presence of ISO. Therefore, in a clinical setting where β-blockers are applied to reduce the heart rate and Ca^{2+} over- load, antiarrhythmic action of $I_{Cl(Ca)}$ could

be even more pronounced as $I_{Cl(Ca)}$ reduces APD heterogeneity at slow heart rates more effectively.

Effects of BAPTA-AM on action potential duration

In the present study, we have clearly shown that the frequently used Ca^{2+} chelator BAPTA-AM effectively and reversibly blocked I_{Kr} in canine ventricular myocytes. Our results are in an excellent agreement with the results of Tang *et al*., who reported an IC_{50} value of 1.3 μM for BAPTA-AM on hERG channels (and similar values for other K^+ channels) expressed in HEK 293 cells. These authors also reported an open channel block with BAPTA-AM. Therefore it is somewhat surprising that a similar degree of block was observed under conventional and action potential voltage clamp conditions (reduction to 32 and 34% of the control values, respectively). Probably the consequences of the more positive activation voltage of +40 mV, applied in conventional voltage clamp, compared to the less positive action potential plateau in action potential voltage clamp experiments, was offset by the higher stimulation frequency of 1 Hz used in the latter experimental design. The most important message of both voltage clamp experiments is that external BAPTA-AM must not be used as an experimental Ca^{2+} chelator when studying the Ca^{2+} dependence of cardiac ion currents in whole cell systems expressing hERG channels, because of the direct inhibitory effect of BAPTA-AM on I_{Kr} current.

Based on the present results, the exposure to BAPTA-AM displays a very complex pattern of changes in APD_{90}, so explanation of the results is necessarily speculative. The rapidly developing acute effect of BAPTA-AM, the significant prolongation of APD_{90}, is very likely due to the BAPTA-AM- induced inhibition of I_{Kr}, an effect evident within the initial 5 min of exposure. This explanation apparently contradicts to the finding that the acute lengthening effect of BAPTA-AM was reduced by nisoldipine and augmented by BAY K8644, which might suggest some kind of involvement of $I_{Ca,L}$ in the concomitant changes of APD_{90}. However, not only $I_{Ca,L}$ was increased or suppressed by BAY K8644 and nisoldopine, respectively, but also the pre- BAPTA value of APD_{90} as well as the level of the plateau potential. Consequences of the I_{Kr}-blockade strongly increases when APD_{90} is longer, as was the case in the presence of BAY K8644, and decreases if APD_{90} is shorter, as was observed in the presence of nisoldipine. In addition, the relative importance of I_{Kr} in regulation of APD_{90} strongly increases with positive shifts in the plateau potential due to the faster activation of I_{Kr} at more positive voltages. This may also contribute to the stronger BAPTA-AM-induced prolongation in the presence of BAY K8644 versus the smaller change observed in nisoldipine.

When the I_{Kr}-blocking effect of BAPTA-AM was prevented by pretreatment of the cells with I_{Kr} blocking drugs, like dofetilide or E 4031, BAPTA-AM initially shortened APD_{90}, an effect likely related to the reduction of $[Ca^{2+}]_i$. The most plausible explanation for this BAPTA-AM-

induced shortening is the conversion of the normally inwardly directed Na^+/Ca^{2+} exchange (NCX) current to an outwardly directed one as a consequence of the reduced $[Ca^{2+}]_i$. In line with this, the tendency of APD-shortening observed after the initial BAPTA- AM-induced prolongation in myocytes pretreated with BAY K8644 was also pronounced probably because the long APD_{90} and the positive plateau potential favored the reverse mode activity of NCX. This effect is in line with the finding that buffering $[Ca^{2+}]_i$ significantly shortened APD_{90} in rat, guinea pig and ferret cardiomyocytes and with own unpublished results, obtained in guinea pig ventricular cells, indicating that net NCX current was outward when recorded with EGTA-containing patch pipettes.

The BAPTA-AM-induced APD_{90}-shortening effect was transient. In other words, there was a progressive tendency of prolongation during a long-lasting exposure to BAPTA-AM. This was also evident under control conditions, where no transient shortening was observed (only a small notch after 5 min). The final lengthening tendency of APD_{90} is likely related to the combination of two further effects of progressive buffering of $[Ca^{2+}]_i$. These are the augmentation of the $I_{Ca,L}$ due to the reduction of its Ca^{2+}- dependent inactivation and offsetting some Ca^{2+}-dependent outward currents, like the Ca^{2+}-dependent Cl^- current by buffering $[Ca^{2+}]_i$. In summary, buffering of $[Ca^{2+}]_i$ resulted in a biphasic response of APD_{90}: a transient initial shortening was followed by progressive prolongation when the I_{Kr}-blocking effect of BAPTA-AM was prevented by the pretreatment with dofetilide or E 4031.

Effect of TRPM4 inhibitor 9-phenanthrol on major ion currents producing action potential

In the present work, effects of the TRPM4 channel inhibitor 9-phenanthrol were studied on action potential configuration and the major underlying ionic currents in canine ventricular myocytes. All voltage-clamp experiments were performed with patch pipettes containing 10 mM BAPTA to prevent the effects of 9-phenanthrol on the Ca^{2+}-dependent TRPM4 channels. The 3 major K^+ currents studied (I_{to1}, I_{Kr}, and I_{K1}) were suppressed in a concentration-dependent manner by 9-phenanthrol, while $I_{Ca,L}$ was not affected. These results seem to differ from those obtained in murine ventricular myocytes, where 10 μM 9-phenanthrol had no effect on $I_{Ca,L}$ and whole K^+ current (I_K) but 100 μM of the compound caused significant inhibition of these currents. In our experiments, $I_{Ca,L}$ was not affected by 9-phenanthrol up to the concentration of 30 μM. On the other hand, canine K^+ channels are more sensitive to the compound than murine ones, because I_{to1}, I_{Kr}, and I_{K1} were already significantly inhibited by 10 μM of 9-phenanthrol in our experiments. Although these ionic currents are different from the murine I_K in several aspects, the results suggest interspecies differences. However, the effect of 9-phenanthrol in human cardiomyocytes has not been investigated yet. Most importantly, our results clearly indicate that 9-phenanthrol is not selective for TRPM4 channels in canine ventricular myocardium. It is highlighted by the IC_{50} value reported for TRPM4 inhibition at 17 and 21 μM, which is in the concentration range for significant K^+ current blockade.

Our action potential recordings revealed that 9-phenanthrol reduced the action potential amplitude, the overshoot potential, and the velocity of depolarization (V^+_{max}). These effects are likely the consequences of 9-phenanthrol-induced Na^+ channel blockade. As Ca^{2+} current was not affected by the compound, suppression of the late Na+ current may also explain the plateau depression observed with 9-phenanthrol. Similarly, the decreased slope of phase 1 and terminal repolarization (V^-_{max}) can easily be explained by suppression of I_{to1} and I_{K1}, respectively. The aforementioned action potential parameters are determined mostly by the action of one ionic current. In contrast, APD is affected by many different ionic currents. Therefore, when discussing the effects of 9-phenanthrol on APD_{90}, many different aspects must be considered. In some cells, significant and reversible shortening was observed, while in others the lengthening of the action potential was observed. As 9-phenanthrol is likely to modify both inward and outward ionic currents (see above), individual cell-to-cell differences in each ionic current density might be responsible for these differences. These results also conflict with data obtained in murine ventricular myocardium, because neither V^+_{max} nor action

potential amplitude was de- creased by 100 μM 9-phenanthrol in that preparation — in contrast to our results. Interestingly, APD was only slightly shortened in murine ventricular, but markedly in atrial cells, where both the amplitude and duration of action potentials were significantly reduced by the drug. A study also suggests that the effect of 9-phenanthrol is more pronounced in atrial than in ventricular myocardium (at least in mice) due to the less abundant TRPM4 channel expression in the ventricles. Here is to be mentioned that TRPM4-knockout murine atrial cells developed shorter action potentials than similar cells obtained from wild type animals. In a study of rabbits, 9-phenanthrol shortened the action potential of Purkinje fibers but not of ventricular strips. In contrast to our results, 30 μM 9-phenanthrol did not change any examined action potential parameters of rabbit ventricular strips. It must be noted, however, that unlike in voltage-clamp experiments where BAPTA prevented the activation of TRPM4 channels, it was not the case in sharp microelectrode action potential recordings. Therefore, the actions of 9-phenanthrol on action potential parameters could include those derived from the inhibition of TRPM4 channels. As TRPM4 is a nonspecific monovalent cationic channel, its reversal potential is around 0 mV. At the initial part of the action potential, intracellular Ca^{2+} concentration is high enough to activate the current moving the membrane voltage towards 0 mV. Therefore, the expected result of the inhibition of TRPM4 channels by 9-phenanthrol is the slowing of phase 1 repolarization, which was actually detected in our experiments. Regarding the effect on later phases of the action potential, one might speculate that by inhibiting TRPM4 channels it is easier for the membrane voltage to move away from 0 mV. This would result in the elevation of the plateau voltage if it was initially positive, and a further decrease in case of negative values. In our measurements, $plateau_{50}$ values were reduced by 9-phenanthrol applied in concentrations of 3 μM and higher in all cells according to what can be expected. Regarding APD, one might expect that the inhibition of TRPM4 could lead to action potential shortening as it was detected in rabbit Purkinje fibers and TRPM4-knockout murine atrial cells. However, 9-phenanthrol had no effect on rabbit ventricular cells and, as mentioned above, APD was only slightly shortened in murine ventricular cells probably due to the less abundant TRPM4 channel expression in the ventricles. Because TRPM4 expression in canine myocardium has not been demonstrated yet, the effect of 9-phenanthrol on action potential parameters in our canine model might be mainly due to the actions of the drug on ion channels other than TRPM4. Even if TRPM4 is expressed in canine ventricular myocardium, one has to keep in mind that the intracellular Ca^{2+} concentration during later phases of the action potential is certainly smaller.

Therefore, that might not cause large enough activation of TRPM4 channels so that a significant effect on APD would be detected in the presence of 9-phenanthrol. APD, however, depends on many ionic currents that might be altered by earlier phases of the action potential, so it is very difficult to predict its change. APD_{90} was the only parameter in which the 9-phenanthrol-induced changes were not uniform in all studied cells. The reason for this might be the different action potential shape of the cells in control conditions. In those cells that responded with action potential shortening, the smaller voltages of phase 1 and $plateau_{50}$ could have prevented the aforementioned plateau voltage increasing action of the drug (assuming the blockade of TRPM4 channels) and, therefore, the terminal repolarization could took place earlier leading to shorter action potentials. According to our previous publication, the longer the action potential, the greater change is expected from any intervention changing APD. These differences in baseline APD might also contribute the different action of 9-phenanthrol on APD, as it was longer in cells where significant shortening could be detected.

The last issue to be discussed is the appearance of a steady outward current at voltages positive to –40 mV in the presence of 9-phenanthrol, which was evident in each individual experiments. As we used 10 mM BAPTA intracellularly to prevent Ca^{2+}-dependent TRPM4 channel activation, this steady outward current cannot be related (at least theoretically) to the effect of 9-phenanthrol on TRPM4 channels. In addition, its reversal potential, which is close to –40 mV is far from the close to 0 mV reversal potential of the TRPM4 current. We can only speculate on the ionic nature of this current. Inhibition of the late Na^+ current might cause an outward shift in the current–voltage relation and thus may contribute to the outward current shift. Activation of a K^+ current is not likely, because I_{K1}, I_{Kr}, and I_{to1} were suppressed by 9-phenanthrol and I_{Ks} was blocked by HMR-1556 during the experiments. Of course, activation of an unidentified K^+ current cannot fully be ruled out. A further possibility for the extra outward current can be the activation of a Cl^- current. The calculated reversal potential for Cl^- in our experimental setting is –32 mV. It worth mentioning, however, that 9-phenanthrol inhibits TMEM16A Cl^- channels, although the effect of 9-phenanthrol is unlikely to be mediated by Ca^{2+}-sensitive TMEM16A channels because of the presence of BAPTA in our pipette solution. However, activation of another Cl^- channel may also be considered. One possible candidate might be the CFTR Cl^- channel, which was suggested to share common structural elements with TRPM4, although the presence of isoproterenol-induced Cl^- current was debated in canine. Further experiments are required to elucidate this point.

The most important conclusion of the present work is that 9-phenanthrol cannot be

considered as a selective inhibitor of TRPM4 channels (at least in canine ventricular myocardium). Therefore, its application as a TRPM4 blocker can only be appropriate in expression systems, in absence of other cardiac ion channels, but not in native cardiac cells.

New scientific results included in the dissertation

Examining the role of intracellular Ca^{2+} concentration in the regulation of ion currents, I obtained the following new scientific results:

1. I characterized the role of $I_{Cl(Ca)}$ in the action of different left ventricular canine cardiac cells with the help of 9-AC (anthracene-9-carboxylic acid) in the development of action potential and its possible arrhythmic effect.

2. I investigated the effect of intracellular Ca^{2+} on the shape of left ventricular myocytes in AP and I_{Kr} (hERG) currents, and showed that the reduction of intracellular Ca^{2+} by BAPTA-AM could modify the characteristics of AP by inhibiting the I_{Kr} current.

3. I characterized the effect of 9-phenanthrol, a specific antagonist of the TRPM4 channel, on a number of ionic currents expressed in cardiomyocytes.

Summary

Oscillations of calcium levels are observed in cardiomyocytes in parallel with changes in transmembrane potentials. A wide range of ion currents in the heart are known to be Ca^{2+}-dependent. In cellular electrophysiological experiments, intracellular calcium concentration is often buffered by a so-called calcium chelators to study Ca^{2+}-dependent ion currents. In some experiments, the cell-permeable, acetoxymethyl ester form of the Ca^{2+} chelating agents are used to reduce $[Ca^{2+}]_i$. Such experiments have been conducted for decades and the results obtained have been interpreted solely by $[Ca^{2+}]_i$ buffering despite the fact that BAPTA-AM interacts with K^+ channels expressed in HEK cells.

We investigated the role of $I_{Cl(Ca)}$ in the spatial and temporal heterogeneity of cardiac left ventricular repolarization by conventional microelectrode technique, conventional and action potential voltage clamp (APVC) technique, and protein expression determination. We also aimed to investigate whether BAPTA-AM influences left ventricular myocardial action potential (AP) and I_{Kr} current. We wanted to investigate the role of the TRPM4 current in the formation of AP morphology and the behavior of the current under AP by action potential clamp measurements with 9-phenantrol. As this method requires as much selective ion channel blocking agent as possible, it was first of all designed to perform the selectivity test for 9-phenanthrol.

Our results show that $I_{Cl(Ca)}$ reduces regional (transmural and apico-basal) and temporal (short-term variability in AP length) repolarization, suggesting that current has an antiarrhythmic role. Neither the density of $I_{Cl(Ca)}$ recorded with the rectangular pulses nor the normalized expression of TMEM16A and Bestrophin-3 proteins differed significantly between the cells of different origin. The $I_{Cl(Ca)}$ density measured by APVC in subepicardial cells was higher than in subendicardial cells, probably due to the greater Ca^{2+} influx by LTCCs in subepicardial APs.

Our studies demonstrated the inhibitory effect of BAPTA-AM I_{Kr} on ventricular myocardial cells.

Our experiments have shown that 9-phenathrol is not a selective TRPM4 channel blocker in canine ventricular myocytes. Therefore, the use of 9-phenanthrol as a TRPM4 inhibitor is desirable only in expression systems and not in native cardiac cells.

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List of publications related to the dissertation

1. Horváth, B., Szentandrássy, N., **Veress, R.**, Baranyai, D., Kistamás, K., Almássy, J., Tóth, A., Magyar, J., Bányász, T., Nánási, P. P.: Effect of the intracellular calcium concentration chelator BAPTA acetoxy-methylester on action potential duration in canine ventricular myocytes.
J. Physiol. Pharmacol. 69 (1), 99-107, 2018.
DOI: http://dx.doi.org/10.26402/jpp.2018.1.11
IF: 2.544

2. **Veress, R.**, Baranyai, D., Hegyi, B., Kistamás, K., Dienes, C., Magyar, J., Bányász, T., Nánási, P. P., Szentandrássy, N., Horváth, B.: Transient receptor potential melastatin 4 channel inhibitor 9-phenanthrol inhibits K+ but not Ca2+ currents in canine ventricular myocytes.
Can. J. Physiol. Pharmacol. 96 (10), 1022-1029, 2018.
DOI: http://dx.doi.org/10.1139/cjpp-2018-0049
IF: 2.041

3. Hegyi, B., Horváth, B., Váczi, K., Gönczi, M., Kistamás, K., Ruzsnavszky, F., **Veress, R.**, Izu, L. T., Chen-Izu, Y., Bányász, T., Magyar, J., Csernoch, L., Nánási, P. P., Szentandrássy, N.: Ca2+-activated Cl- current is antiarrhythmic by reducing both spatial and temporal heterogeneity of cardiac repolarization.
J. Mol. Cell. Cardiol. 109, 27-37, 2017.
DOI: http://dx.doi.org/10.1016/j.yjmcc.2017.06.014
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List of other publications

4. Kristóf, E., Klusóczki, Á., **Veress, R.**, Shaw, A., Combi, Z., Varga, K., Győry, F., Balajthy, Z., Bai, P., Bacsó, Z., Fésüs, L.: Interleukin-6 released from differentiating human beige adipocytes improves browning.
   Exp. Cell Res. 377 (1-2), 47-55, 2019.
   DOI: http://dx.doi.org/10.1016/j.yexcr.2019.02.015
   IF: 3.329 (2018)

5. Horváth, B., Szentandrássy, N., **Veress, R.**, Almássy, J., Magyar, J., Bányász, T., Tóth, A., Papp, Z., Nánási, P. P.: Frequency-dependent effects of omecamtiv mecarbil on cell shortening of isolated canine ventricular cardiomyocytes.
   Naunyn Schmiedebergs Arch. Pharmacol. 390 (2), 1239-1246, 2017.
   DOI: http://dx.doi.org/doi: 10.1007/s00210-017-1422-z
   IF: 2.238

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