PH.D. THESIS

Regional and sexual inhomogenity in the mammalian ventricular myocardium

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I. INTRODUCTION

The muscle of the heart according to the traditional point of views is a „functional syncytium" composed of cells of homogenous morphology and function. This dogmatic approach was soon diminished after the first discoveries of the functional differences of the cells located in the right and left ventricules. Soon new observations were found regarding the ventricular inhomogenity and nowadays it is a lecture book data. The morphology of the action potentail shows marked differences between the atria and the ventricule, between the right and left ventricule or in the layers of the myocardium. The differences of the action potentials in the regions of the heart are caused by the regional inhomogenity of the ionic currents of the membrane. In this work we wanted to observe if the electrophysiological differences of the cardiac cells can be explained by the different patterns of expression of the ion channels.

I/1. Inhomogenity in the layers of the myocardium

Ventricular myocardium was initially thought to be homogeneous First distinctions has been made in the action potential morphology among different layers of ventricular myocardium. Action potentials of the easily separable surface layers were first compared resulting in characterization of the endocardial and epicardial myocytes. As the experimental techniques improved cells with different electrical properties, were identified buried in the free wall of canine ventricular myocardium (midmiokardium), the M cells.

Transmural heterogeneity of the ventricular action potential (AP) has been described in detail in many species, including dog, pig, guinea-pig, rabbit, rat and human. The differences in the layers of the ventricular wall of the Na^+-current (I_{Na}) that is responsible for the upstroke of the action

potential has also been observed in many species. When the amplitudes of action potentials are compared according to the transmural position of myocytes in the various species, the results are quite controversial. Taken into account all the results of the publications regarding the AP amplitude the following tendency seems to emerge: epicardial cells display smaller action potential amplitude than endocardial ones.

A prominent and early fast repolarization in phase 1 and the subsequent depolarization together known as spike-and-dome configuration is evident only in epicardial and M cells, but is absent or hardly detectable in endocardial cells. This spike-and-dome configuration is most prominent in canine, less detectable in human, porcine and rabbit, and totally missing from guinea-pig action potentials In canine ventricular cells, phase 1 magnitude is smaller in endocardial cells than in the other two cell types, and is greater in epicardial than in M cells. On the level of ionic currents I_{to} has been described to be higher in epicardial and midmyocardial cells than in endocardial ones in many examined species including murine, rabbit, rat, canine and human myocytes. Between the epicardial and the M cells the epicardial ones had significantly larger I_{to} amplitude.

After the plateau phase of the action potential the I_K and the I_{K1} will repolarize the membrane creating the 3. phase of the action potential. The height of plateau phase or the phase 2 amplitude has an effect on the final repolarization. The higher is its amplitude, the rapid delayed rectifier potassium current (I_{Kr}) activates faster, thereby initiating an earlier final repolarization and resulting in a shorter action potential. This is the case in epicardial cells where phase 2 amplitude is the highest, and their action potential is the shortest according to the majority of relevant reports. Shorter epicardial than endocardial action potentials were recorded in canine, porcine, guinea-pig and rabbit myocytes. M cells have clearly the

longest action potentials and their rate dependence is the steepest compared to the epi- and endocardial ones, namely their action potential lengthens at the largest extent when increasing the pacing cycle length.

The publications regarding the ionic currents of the rabbit responsible for the terminal repolarization of the AP state that smaller I_{Ks} but similar I_{Kr} density in midmiocardial cells compared with epicardial cells was described in good agreement with the higher M cell *versus* epicardial action potential duration. Shorter action potentials and higher I_{Ks} (but not I_{Kr}) density with similar kinetic properties were found in epicardial than in endocardial myocytes. Several studies reported transmural differences in delayed rectifier potassium currents in canine left ventricular myocytes as well. The smallest I_{Ks} density (with no differences in its kinetic properties), in agreement with the longest action potentials, were observed in midmyocardial cells. No transmural difference in the density or voltage dependence of the inward rectifier potassium current (I_{K1}) has been found in any of the examined mammalian species.

The physiological significance of the transmural gradient caused by the epicardial/endocardial differences is the generation of the postiv direction of the repolarizing T and (supposedly) U wave on the ECG record.

I/3. Vertical inhomogenity in the heart muscle

In contrast to the previously discussed transmural differences much less papers have been published in the topic of the vertical inhomogenity. The apico-basal differences have been examined in many species including canine, rabbit, rat, ferret, porcine, guinea-pig and human hearts.

Casis et al. compared action potential duration on rat between cells isolated from the epicardium of the apex and the endocardium of the base

and found that the AP of the apical cells were shorter than the basal ones thus the reported vertical differences might be distorted by the transmural gradient. In guinea-pig, Watanabe et al. failed to explore apico-basal asymmetry in left ventricular multicellular epicardial preparations. However, shorter action potentials were recorded in the apical than basal regions of the right ventricular endocardium and left ventricular septum. In humans longer, as well as shorter action potentials were recorded from the apical than basal regions of the left ventricular epicardial surface.

In summary we can conclude that there is large amount but sometimes controversial data available in literature regarding the regional inhomogenity of the action potential parameters and the underlying ionic current caracteristics. Due to the marked interspecies differences in action potential configuration and the underlying ion currents found among the most frequently used laboratory animals, results obtained in human samples have exceptional importance.

The aim of our study was to systematically map the transmural and vertical inhomogenity of the mammalian myocardium with electrophysiological methods. We were curious if the found functional differences are correlated to the to the ion channel expression patterns.

I/4. Effects of sex hormones on the electrical properties of the myocardium

Differences can be found not only between the different regions of one heart but between different hearts of the same species. It is obvious to examine the gender-related differences because there are well known differences between for example the male and female ventricular repolarization.

As the changes in the AP parameters and ionic currents can be well observed in a surface ECG recording first we can compare electrical

properties of different hearts on ECG records. If differences can be seen it can be assumed that the electrophysiology parameters and the ion channel expression levels of the myocardium has also differences.

These sexual differences – based on comparative ECG studies performed in normal and castrated men and women – seem to be primarily related to the presence or absence of testosterone, and only moderately influenced by female sexual steroids. The mechanism of cardiac effects of testosterone and oestrogen is poorly understood, therefore, an animal model suitable for studying the molecular background of these actions would be straightforward.

As substitution of sex hormones is currently applied in the clinical practice, the present work was designed to study the effects of oestrogen and testosterone on the ECG parameters in dog in order to compare the results with those obtained in humans.

We found our canine model reasonably suitable (although with limitations) for further investigations on the actions of sex hormones, and to draw conclusions relevant for human.

II. OBJECTIVES

The aim of present work was to correlate the electrophysiological inhomogenity of the mammalian ventricular moycardium in one heart and between different hearts of the same species to the differences observed in the ion channel expression patterns.

III. MATERIALS AND METHODS

III/1. Cardiac preparations

The hearts of adult mongrel dogs of either sex breed for experimental purposes were used for our study. Human ventricular tissues

were dissected from 7 undiseased donor hearts stored in cardioplegic solution. The hearts were obtained from general organ donor patients whose semilunar valves were used for transplantation. In cases of both human and canine hearts approximately 5x5 mm segments were dissected from the apical and the basal regions of the heart of the left ventricular free wall then a dermatome was used to peal off very thin (having width less than 0.5 mm) subepicardial strips from the region located halfway on the apico-basal axis of the left ventricle. Similar samples were also dissected from the central layer of midmyocardium (typically 3 mm underneath the epicardium) for determination of channel protein density.

III/2. Electrophysiological studies

III/2. 1. Isolation of cells

Single canine myocytes were obtained by enzymatic dispersion using the segment perfusion technique.

III/2. 2. Measuring action potentials

Measuring membrane potential we used the single cell method described by Volders et al. Action potentials were recorded from isolated cells superfused with normal Tyrode solution at 37 ^{o}C. Transmembrane potentials were recorded using glass microelectrodes filled with 3 M KCl and having tip resistance between 25 and 30 MΩ. During every experiment we averaged 10 concomittant AP and determined the resting membrane potential, the maximal rate of depolarisation (V_{max}), the AP amplitude and the duration of the action potential at the 20, 50 and 90% of its repolarization (APD_{20}, APD_{50} és APD_{90}).

III/2. 3. Ionic currents measurements with voltage-clamp technique

Whole cell configuration of the patch clamp technique was used for recording transmembrane ion currents in normal Tyrode solution at 37 $^{\circ}$C.

III/3. ECG recordings

THe ECG recordings were done on ten male and 10 female anesthetized adult mongrel dogs of sexually active age (2–4 years) breed for experimental purposes The basic ECG parameters (heart rate, PQ interval, QRS duration and QT interval) were continuously monitored on a 12 channel PC-controlled electrocardiograph. After taking control records the animals were challenged with dofetilide (HERG channel inhibitor).

When completing measurements both male and female dogs were castrated. A period of 1 month was allowed for stabilization of the new endocrine status of the animals, and following this recording of the baseline ECG and application of dofedilide were repeated. Finally, the animals received inverse hormonal substitution for 4 weeks (i.e. castrated males were injected with 17b-estradiol-benzoate, while females were treated with 5a-dihydroxytestosterone-propionate, and ECG recording – including the dofetilide challenge – was repeated again.

At each stage of the experiment blood samples of 3 mL were taken before the application of dofetilide. 17b-estradiol and testosterone levels from the samples were determined with an electro-chemiluminescence immunoassay technique (ECLIA-kit). After completing the protocol above, small transmural sections from the left ventricular free wall of each animal (i.e. from testosterone-treated castrated females and oestrogen-treated castrated males, as well as from untreated male and female dogs) were dissected and used for determination of channel protein densities.

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III/4. Western (immuno)blot

Membrane proteins from the different regions of canine and human left ventricles were obtained using a method modified after Han et al.The following primary antibodies were used: rabbit anti-Kv1.4, anti-Kv4.3, anti-Kir2.1, anti-Nav1.5, anti-α_{1C}, anti-minK, anti-HERG and anti-MiRP1, and goat anti-KChIP2 and anti-LQT1.

III/5. Statistics

The arithmetic means and the standard error of mean were calculated from our results. Statistical significance was determined using one-way ANOVA and Student's t-test. Differences were considered significant when the P value was less than 0.05.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and with the principles outlined in the Declaration of Helsinki. The experimental protocol, applied for human hearts, was also approved by the local ethical committee (No. 51-57/1997 OEJ).

IV. RESULTS

IV/1. Action potential characteristics and membrane capacitance

During the experiments the action potential parameters were compared on cells originated from apical (APEX), basal (BASE) and epicardial (EPI) and midmiocardial (MID) regions of the heart. In the APEX-BASE comparison the cells were digested from the midmiocardial layer of the heart. Action potential of EPI and APEX cells were shorter in duration compared to the MID and BASE ones. The APs of EPI and APEX cells displayed more prominent early (phase-1) repolarization than MID and BASE ones. In the same time MID cells had faster maximum

velocity of depolarization (V_{max}) and their AP amplitude was also larger. No significant differences were observed in the maximum rate of depolarization and in the action potential amplitude of the cells originated from the apical and basal regions. There was also no difference in the magnitude of the resting potential between the examined regions.

The cell capacitance (determined in voltage clamp mode) was practically equal in all the myocytes suggesting that the size of these cells was similar.

IV/2. Inward rectifier potassium current (I_{K1})

The steady-state current-voltage relationship of the membrane was obtained in voltage clamp mode by applying 400 ms long test pulses to potentials ranging between –130 to +50 mV in the presence of 5 μM nifedipine. The average values of the current amplitudes obtained from different regions of the heart were not significantly different in the -130 +40 mV voltage range.

IV/3. L-type calcium current (I_{Ca})

During L-type calcium current measurement Tyrode solution was supplemented with 3 mM 4-aminopyridine, 1 μM E 4031, and 30 μM chromanol 293B in order to block K^+ currents. The current amplitude, the voltage dependance of the amplitude and the inactivation and the time constant of inactivation measured at +5 mV were determined. There was no significant difference in the examined I_{Ca} parameters in any examined region or layer of the myocardium. It can be concluded that the calcium currents were similar in every examined part of the mammalain heart.

IV/4. Transint outward potassium current (I_{to})

I_{to} was activated by depolarizations of 400 ms duration to test potentials ranging from –20 to +60 mV, arising from the holding potential

of –80 mV and increasing in 10 mV steps. Before each test pulse a short (5 ms) depolarization to –30 mV was applied in order to inactivate the fast Na^+ current, while Ca^{2+} current was blocked by 5 μM nifedipine. EPI and APEX myocytes displayed significantly larger peak current density than MID and BASE ones at test potential higher than +10 and +20 mV.

Voltage dependence of inactivation was determined using test depolarizations to +50 mV following a set of prepulses clamped to various voltages between -70 and -10 mV for 500 ms in 5 mV proportions, the holding potential was -80 mV. Peak currents measured after these prepulses were normalized to the peak current measured without prepulse and plotted against the respective prepulse potential. Data were fitted to the Boltzmann function

No significant differences were observed when comparing voltage dependence of inactivation in EPI and MID myocytes The steady-state inactivation curve obtained for I_{to} in apical myocytes was shifted by 4 mV to the right comparing to the curve determined for basal cells. In addition, the 'apical' curve was slightly, but significantly, steeper than the 'basal' one.

Time constant of inactivation of I_{to} was measured at +50 mV by fitting the decaying limb of the current recorded to an exponential function. No significant differences were observed between any examined cell groups in the time constant of inactivation.

IV/5. Rapid component of the delayed rectifier potassium current (I_{Kr})

A I_{Kr} was activated by sets of 200 ms long depolarizing pulses clamped to test potentials ranging from –20 to +40 mV. I_{Kr} was assessed as tail current amplitudes recorded following repolarization to the holding potential of –40 mV. I_{Ca} and I_{Ks} were suppressed by 5 μM nifedipine and 30 μM chromanol 293B, respectively. The amplitudes of the I_{Kr} current

tails were not significantly different in the myocytes of apical and basal origin when the current was activated at +40 mV, however, within a narrow range of activation voltage (around +10 mV) apical tail currents were smaller in amplitude than basal ones. The voltage dependence of activation of I_{Kr} was determined by plotting tail current amplitudes against the test potential used to activate the current. These activation curves were fitted to the two-state Boltzmann function. The results indicate that I_{Kr} requires less positive potentials to activate in basal cells than in apical ones.

Time constant for activation of I_{Kr} was determined using the tail envelope test by applying depolarizations to +30 mV with durations continuously increasing from 5 to 900 ms. The amplitudes were plotted in the function of the duration of the pulse and fitted with a monoexponential function. Activation time constants were not different significantly in in apical and basal myocytes. Deactivation time constants were measured by fitting the decaying current tails as a sum of two exponential components. No significant differences were seen between the faster or slower time constants obtained for deactivation of I_{Kr} in apical and basal cells, respectively

During the comparison of the different layers of the myocardium we determined the voltage dependance of the activation but found no significant differences between the EPI and MID cells. Activation time constants were not different significantly in EPI and MID myocytes Deactivation of I_{Kr} was faster in the EPI cells: both the rapid and slow deactivation time constants were significantly shorter in EPI than in MID myocytes.

IV/6. Slow component of the delayed rectifier potassium current (I_{Ks})

I_{Ks} was evaluated as a fully activated current as well as a tail current Test depolarizations, arising from the holding potential of –40 mV and having duration of 3 s, were varied from –30 to +50 mV. I_{Ca} was inhibited by 5 µM nifedipine and I_{Kr} was blocked using 1 µM E 4031. Both the fully activated I_{Ks} and its tail current were significantly larger in apical than in basal myocytes. In contrast to results obtained with I_{Kr}, both time constants (i.e. those estimated for activation and deactivation of I_{Ks}) were significantly shorter in apical than in basal cells. Time constant for activation was determined using the tail envelope test. The time constants were significantly shorter in case of the apical cells compared to the basal ones. Deactivation of I_{Ks} was measured at membrane potentials ranging between –40 mV and +30 mV, following activation at +50 mV for 3 s. Deactivation of I_{Ks} was voltage dependent and followed monoexponential kinetics. The time constant was significantly shorter in apical than in basal cells within the voltage range of –10 - +20 mV.

Both the fully activated I_{Ks} and its tail current were significantly larger in EPI than in MID myocytes. In addition, no difference in the voltage-dependence of activation was observed in the two cell types. Deactivation of I_{Ks} was best fitted as a sum of two exponential components. No significant differences were observed in the faster or slower time constant between the EPI and MID cells

IV/7. Regional distribution of ion channel proteins

It was reasonable to test whether the observed apico-basal differences in ion current densities are indeed due to true differences in expression of various channel proteins. Therefore, expression of the underlying channel forming proteins (α-subunits), together with some of their important regulatory subunits, was compared in pairs of left

ventricular myocardiac tissue chunks excised from the apical and basal regions and from the layers of epicardium and midmiocardium of 8 canine hearts. According to our co-operation with the Department of Pharmacology and Pharmacotherapy from the University of Szeged we had the opportunity to examine samples obtained under similar conditions from 5 undiseased human hearts.

We found that expression of α_{1C} (pore-forming subunit of L-type Ca^{2+} channel), Kir2.1 (pore-forming subunit of the inward rectifier K^+ channel), as well as HERG and MiRP1 (pore-forming and regulatory subunits of the I_{Kr} channel, respectively) was not significantly different in the apical and basal canine ventricular tissues. In contrast, expression of KvLQT1 and MinK (pore-forming and regulatory subunits of the I_{Ks} channel) was significantly less in basal than in apical myocardium. Similar asymmetry was observed in distribution of channel proteins responsible for mediation of I_{to}: the expression of Kv1.4 and KChIP2 (pore-forming and regulatory subunits, respectively) was significantly lower in the basal than in the apical region of the canine heart. The expression of Kv4.3 (another type pore-forming subunit of I_{to}) was also less in basal than apical tissues, however, this difference failed to reach the level of statistical significance. Although quantitative differences in the expression of KChIP2, MinK and Kv1.4 were observed between dog and human, the pattern of apico-basal asymmetry was similar in the two mammalian species.

Levels of α_{1C} (pore-forming subunit of L-type Ca^{2+} channel), Kir2.1 (pore-forming subunit of the inward rectifier K^+ channel), as well as HERG and MiRP1 (pore-forming and regulatory subunits of the I_{Kr} channel, respectively) was not significantly different in the EPI and MID regions of canine myocardium. In contrast, significant differences were observed in distribution of channel proteins responsible for mediation of

I_{to}: the density of Kv4.3, Kv1.4 and KChIP2 (two pore-forming and one regulatory subunit, respectively) was significantly lower in the MID than in the EPI region. Density of KvLQT1 and MinK (pore-forming and regulatory subunits of the I_{Ks} channel) was also asymmetrical: density of KvLQT1 in the EPI, while that of MinK in the MID region was more pronounced. In addition, the density of Nav1.5 (pore-forming subunit of the fast Na^+ channel) was twice higher in the MID than in the EPI region.

We have results obtained under similar conditions in samples excised from 5 undiseased human hearts. Although smaller quantitative differences in the density of some channel proteins could be observed between dog and human, the pattern of EPI-MID asymmetry was basically similar in the two mammalian species – except the density of HERG. Canine EPI and MID regions contained HERG in similar quantities, in sharp contrast with the human heart, where the density of HERG was significantly higher in the EPI than in MID myocardium.

IV/8. Effects of sex hormons on the ECG parameters

In our earlier experiments the inhomogenity of the ion channel expression and their function was examined in the myocardium. Next we wanted to know how the ion channel expression and function changes in animals with different hormone status. Castration and hormon substitution was observed.

IV/8. 1. Plasma hormone levels

Plasma levels of both testosterone and oestrogen were monitored throughout the entire experiment. Castration of male dogs resulted in a reduction of plasma testosterone by one order of magnitude close to the values obtained in control or castrated females. Application of testosterone to castrated females yielded higher testosterone

concentrations in the blood of these animals than that measured in control males. Oestrogen concentrations in the control males and in castrated females were less then the limit of detection of the kit (18,4 pmol L^{-1}) therefore could not be accurately determined in the experimental groups except for the oestrogen-treated castrated males, their blood contained 162 ± 43 pmol L^{-1} of 17b-estradiol. It can be concluded, however, that the inverted hormone substitution was effective in both cases.

IV/8. 2. Heart rate and atrioventricular conduction

If the function of the ion channels changes in the heart or in the sinus or AV node than this change will be visible in the heart rate and in the speed of the atrioventricular conduction (PQ interval). Castration moderately reduced the heart rate in both male and female dogs. These changes were statistically significant in females and marginally significant ($P < 0.1$) in males. Inverse substitution of the sex hormones restored the initial heart rate in both genders. Similar pattern of changes were seen in the PQ interval: castration resulted in significant lengthening of the PQ interval, while values close to the control were obtained following inverse hormonal substitution.

IV/8. 3. Ventricular depolarisation and repolarization

Intraventricular conduction is characterized by the duration of the QRS complex. Neither castration, nor inverse hormone substitution resulted in significant alteration of QRS duration, indicating that the velocity of intraventricular conduction is not much affected by sex hormones.

In contrast to depolarization, ventricular repolarization was markedly affected by the presence or absence of testosterone. Orchiectomy significantly increased both QT and QTc intervals in male

dogs. Injection of oestrogen to the castrated males for 1 month caused some further increases in QT and QTc, but these changes were not significant statistically. Ovarectomy in females had no significant effect on QT or QTc duration. Chronic application of testosterone to these animals significantly shortened repolarization. Orchiectomy increased QT- and QTc-dispersion significantly, which was further increased by application of oestrogen. Similarly to results obtained with the QT and QTc intervals, ovarectomy failed to alter dispersion significantly, but testosterone treatment reduced QTand QTc-dispersion in the castrated females.

IV/8. 4. Dofetilide induced lenghtening or repolarization

Application of dofetilide decreased the heart rate by 20–30 beats min^{-1} in both genders independently of the actual hormonal stage. This bradycardia was always associated with marked lengthening of the QT and QTc intervals. Deprivation of testosterone (castration of male dogs) augmented this lengthening significantly, and administration of testosterone to castrated females significantly reduced the dofetilide induced lengthening. No significant effect of oestrogen could be detected: neither castration of females, nor the oestrogen-injections to castrated males caused any significant change in the lengthening effect of dofetilide.

IV/9. Effects of sex hormones on the expression pattern of channel protein

Expression of channel forming proteins (a-subunits), together with some of their important regulatory subunits, was first compared in tissue samples excised from the ventricular myocardium of testosterone-treated ovarectomized females and oestrogen-treated orchiectomized males. Most

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of the examined ion channel protein expressions were not different in the two groups. In contrast, significant differences were observed in distribution of channel proteins responsible for mediation of I_{K1} and I_{to}: the density of Kir2.1 (pore-forming subunit of the inward rectifier K^+ channel) and Kv4.3 (pore-forming subunit of the channel mediating the major component of I_{to}) was significantly higher in the testosterone-treated ovarectomized females than in oestrogen-treated orchiectomized males. The expression level of Kv1.4 (pore-forming subunit of the other component of I_{to}) was also elevated in the testosterone-treated group, but this difference was only marginally significant. Interestingly, no difference was found in the level of KChIP2, the most important regulatory subunit modulating I_{to}.

In another series of experiments the relative densities of the Kv4.3 and Kir2.1 proteins were directly compared with four simultaneously studied experimental groups: normal (untreated) male and female dogs, testosterone-treated ovarectomized females and oestrogen-treated orchiectomized males. Expression of both Kir2.1 and Kv4.3 proteins was significantly higher in the testosterone-treated castrated females and normal males than in the oestrogen-treated castrated males and normal females. In other words, the presence of testosterone alone was decisive independently of the gender of the animal and the presence or absence of oestrogen.

V. DISCUSSION

In spite of the multitude of studies published on regional and transmural heterogeneity of the mammalian ventricular wall, our knowledge regarding this issue in the healthy human heart is quite incomplete – mostly due to the limited availability of healthy human ventricular tissues. In absence of this we need a reasonably suitable

model to be applied because in the end, the main purpose of basic science is the human. In this study we are first to systematically analyze the regional and transmural asymmetry in the density of various ion channel proteins in healthy human ventricular myocardium comparing them to canine data obtained under identical experimental conditions. Completing the results of Western blot experiments with electrophysiological data in dog may reveal the significance of a channel subunit in generation of the total current and allows comparison of kinetic properties of the current in the different layers of the heart and between different hearts of the same species.

V/1. Regional differences of the myocardium

V/1. 1. EPI/MID assmetry

Using our thin EPI strips, we demonstrated the presence of marked EPI-MID differences in the ion currents of canine myocardium and in the pattern of channel proteins in both canine and human hearts. The density of I_{to} and I_{Ks} currents was found to be much larger (by 55% and 50%, respectively) in the EPI than in the MID region, while no significant difference was seen in the I_{Kr} and I_{K1} densities. With regard to I_{Ks}, our results are congruent with those of other laboratories reporting higher density of I_{Ks} in EPI than in MID canine ventricular cells. The higher EPI/MID ratios of I_{Ks} and I_{to} may result in a shorter and more spike-and-dome shaped action potential of the EPI cell, however, the question arises whether the EPI-MID differences observed in action potential duration can fully be explained by this asymmetrical distribution of I_{Ks} and I_{to}. Probably not. The density of sodium channel protein (Nav1.5) in EPI canine myocardium was only 49% of the value obtained in the MID region. Since the rapid inactivation kinetics of I_{to} and the very slow activation time constant of I_{Ks} may limit the effect of these currents on

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repolarization, it seems likely that the more pronounced Na^+ current in the MID region (indicated by the higher Nav1.5 density, action potential amplitude and V_{max}) may also be responsible for the longer action potential duration of the MID cell.

Similar to results of other laboratories, no EPI-MID differences were observed in the amplitude of I_{Kr} current tails when measured under voltage clamp conditions using the conventionally low stimulation cycle length of 20 s. On the other hand, deactivation kinetics of I_{Kr} was significantly slower in MID than in EPI myocytes, implicating higher amplitude of I_{Kr} in the MID cell at physiological heart rates. This may contribute to the stronger action potential lengthening effect of class 3 antiarrhythmics observed in the MID region of the heart.

Expression of all channel proteins responsible for mediation of I_{to} (Kv4.3, Kv1.4 and KChIP2) was significantly higher in EPI than in MID myocardium – in a good agreement with the electrophysiological data. From these I_{to} channel proteins the density of Kv4.3 showed the best proportionality with peak I_{to} values. At the first glance this is not surprising if bearing in mind that Kv4.3 is the major pore-forming channel subunit in both canine and human ventricular myocardium. However, it is worth of mentioning that Rosati et al. observed only small differences in the expression of Kv4.3 mRNA between EPI and MID canine ventricular myocardium, and the large transmural I_{to} gradient best correlated with the gradient found in KChIP2 mRNA expression. This discrepancy may probably be due to possible differences between the mRNA expression levels and channel protein densities, which are not necessarily identical.

The EPI-MID asymmetry in I_{Ks} revealed by the electrophysiological measurements in canine myocytes shows an excellent coincidence with the EPI-MID distribution of the pore-forming channel subunit KvLQT1,

since the density of KvLQT1 protein and I_{Ks} tail current was uniformly 50% higher in EPI than MID myocytes. In contrast, we found an opposite EPI-MID distribution of the regulatory subunit MinK (i.e. density of MinK in the EPI region was only 65% and 71% of the respective values obtained in canine and human MID myocardium). In other words, the uneven EPI-MID distribution of I_{Ks} seems exclusively to be due to the asymmetrical density of KvLQT1, independently of the actual density of MinK. This seems to be in line with the results of Péréon et al., who failed to demonstrate significant transmural differences in MinK mRNA expression in human cardiac samples explanted due to cardiomyopathy. This issue, however, needs further investigation, since MinK is known to also associate with α-subunits other than KvLQT1.

It has been previously shown that Na^+ current is most prominent in the MID layer of canine myocardium, resulting in higher V_{max} values than obtained in either EPI or endocardial regions. We found 49% EPI/MID ratio in Nav1.5 density, which is close to the value of 59% reported in dog when measuring late Na^+ current under voltage clamp. The 49% EPI/MID ratio obtained for Nav1.5 is apparently far from the 79% ratio determined for V_{max}. Although V_{max} is widely used to characterize Na^+ conductance or I_{Na}, V_{max} is clearly not a linear function of I_{Na}. The extent of this nonlinearity may be a matter of debate, however, a 50% reduction of Na^+ conductance predicts approximately 20% decrease in V_{max}, which is in excellent agreement with the present results.

V/1. 2. APEX/BASE inhomogenity

Our study is first to systematically demonstrate the presence of marked apico-basal differences in ion currents of canine myocardium and the pattern of ion channel proteins in both canine and human hearts. The densities of I_{to} and I_{Ks}, currents contributing to ventricular repolarization

in most mammalian species, were found to be approximately twice larger in apical than in basal myocytes providing reasonable explanation for the shorter apical action potential.

Among the channel proteins responsible for I_{to}, the expression of Kv1.4 and KChIP2 was significantly lower in basal than in apical myocardium. This seems to be congruent with the apical predominance of I_{to}, however, it was previously showed that Kv4.3 is the major pore-forming channel subunit in both canine and human ventricular myocardium. Therefore, the small apico-basal asymmetry observed in Kv4.3 expression fails to explain the large differences seen in the current. Since the modulatory subunit KChIP2 is known to associate with human and canine Kv4.3 subunits, and coexpression of KChIP2 with Kv4.3 increases the amplitude of I_{to}, it is plausible to assume that the higher apical expression of KChIP2 may be responsible for the stronger apical I_{to}. Indeed, Rosati et al. came to same conclusions when studying the transmural I_{to} gradient in dog and human, i.e. that the predominantly epicardial expression of KChIP2, rather than that of Kv4.3, may be the reason for the well-documented epi-encocardial asymmetry of the current. An additional indirect support for such a mechanism comes from the study of Yu et al. demonstrating that the amplitude and kinetic properties of I_{to} could be modulated by angiotensin II and losartan without altering the relative expression of Kv4.3 and Kv1.4 subunits. Although we did not measure I_{to} recovery, decrease of Kv1.4 in the basal region can be expected to accelerate reactivation of the current.

The apico-basal asymmetry in I_{Ks} revealed by the electrophysiological measurements in canine myocytes was corroborated by the Western blot studies performed in both canine and human ventricular tissues, since expression of KvLQT1 and MinK (channel proteins involved in mediation of I_{Ks}) was significantly higher in apical

than in basal myocardium. Contribution of I_{Ks} to normal repolarization has recently been questioned due to its slow activation kinetics, whereas it was supported by other studies. Activation of I_{Ks} was faster by 44% in apical than basal canine myocytes, suggesting that activation of this current may accelerate repolarization or strengthen at least the repolarization reserve in a greater extent in apical cells.

Although no apico-basal differences were obtained in the maximal current density of I_{Kr} (neither in the expression of HERG and MiRP1 proteins), activation of I_{Kr} at 10 mV was weaker in apical than basal myocytes. This relative lengthening, however, seems to be overhelmed by the more pronounced shortening effects of I_{to} and I_{Ks} in apical cells.

V/1. 3. Significance and the limitatons of the study

In the absence of relevant electrophysiological data from healthy human myocardium, only the Western blot results could be compared directly between dog and human. In general, the pattern of EPI-MID inhomogeneity was very similar in the two species, except the distribution of HERG. In dog no EPI-MID differences were found in the density of HERG (nor in the amplitude of I_{Kr}), whereas in human myocardium the EPI/MID ratio of HERG was 1.49. The reason of this difference is unknown, voltage clamp studies performed in healthy human ventricular myocytes of EPI and MID origin may elucidate this point.

In general, the apico-basal inhomogeneity was qualitatively similar in the two species, although quantitative differences could be explored in the expression of KChIP2, MinK and Kv1.4.

Our data suggest that canine heart may be used as a model for studying electrical inhomogeneity in the human heart – but only with limitations. However, considering the large interspecies differences seen

in the literature regarding the apico-basal and the epicardial-midmiocardial gradient, canine ventricular myocardium still appears to be the best human model in spite of the existing quantitative differences. Since the APEX/BASE and EPI/MID inhomogeneity of repolarization, explored in this study, may result in increased prevalence of cardiac arrhythmias due to increased dispersion under certain (usually pathological) conditions and can modulate efficacy of antiarrhythmic drugs, present results may extend our basic knowledge and be utilized when developing more effective and rational antiarrhythmic therapy.

V/2. Sex homrmones and cardiac ion channels

Studying the effects of testosterone and oestrogen, the major sexual steroid hormones in mammals, we have first shown that the two hormones had identical effects on the heart rate and the atrioventricular conduction time. In both cases the pacemaker activity of the sinus node, as well as the conduction velocity in the atrioventricular node was increased by oestrogen or testosterone treatment. Deprivation of sex hormones (castration) had an opposite effect.

The most important result of this study was to show that canine ventricular myocardium – in contrast to nodal tissues – responds in a different way to testosterone and oestrogen: testosterone markedly accelerated ventricular repolarization, decreased the dofetilide-induced lengthening of repolarization, and reduced dispersion in both genders, while oestrogen had a weak opposing or no effect on these parameters. Our results are in a good accordance with those obtained in human ECG studies suggesting that the canine model may be suitable for studying the mechanism of cardiac actions of sex hormones, and to draw conclusions possibly relevant for the human.

As neither castration of female dogs, nor oestrogen treatment of castrated males caused significant changes in ventricular repolarization, it is reasonable to assume, that the differences seen in the ion channel pattern of the inversely hormone-substituted dogs are likely related to the presence or absence of testosterone. This conclusion is further supported by the results of the experiments where the expression of Kv4.3 and Kir 2.1 channel proteins were compared not only between the inversely substituted castrated dogs but also between untreated normal animals. This latter comparison clearly demonstrated a higher expression level of these channel proteins in normal male than in normal female dogs, which finding seems to be in conflict with the practically equal QT and QTc intervals found in the two genders under baseline conditions. It is possible, therefore, that intersex differences may exist in further ion channels, not examined in this study, which might compensate for the higher K^+ channel density in males.

As discussed above, testosterone-treatment facilitated the expression of two K^+ channel proteins (Kir2.1 and Kv4.3) which are responsible for mediation of I_{K1} and I_{to} respectively. I_{K1} is an important current involved in ventricular repolarization, thus its increase may account for the testosterone-induced shortening of QT and QTc intervals. Although the role of I_{to} in normal repolarization of the canine myocardium has recently been questioned, both currents are thought to contribute to the repolarization reserve capacity of myocytes. Therefore, the increased expression of I_{K1} and I_{to} is likely involved in the reduced dofetilide-sensitivity of the testosterone treated myocardium – in spite of the unaltered density of HERG channels. However, the contribution of other K^+ channels which were not studied in the present work (e.g. Kv3.4 or Kv1.7) cannot be excluded.

V/2. 1. Significance and limitations of the ECG studies

In absence of information on the actions of testosterone and oestrogen on ion channels in undiseased human ventricular muscle, we cannot directly compare our Western blot results with relevant human data. Based on the similarities in the cellular electrophysiological properties of the human and canine ventricular cells, and on good coincidence found between the present results and human ECG data, canine ventricular myocardium seems to be a good model for studying the effects of sex hormones. This model, however, has certain limitations. The most striking difference between dogs and humans – at least regarding their sex hormone production – is the 28 days menstrual cycle of women in sharp contrast with the typically anestrus state of female dogs, whose endogenous oestrogen levels were often below the 18.4 pmol L^{-1} detection limit of the ECLIA kit. Therefore, untreated dogs are not really suitable for modeling gender related differences in human, but – especially following castration – the canine model seems to offer the best approach to elucidate the mechanisms of action of sexual steroids.

V/3. Conclusion

During our work we examined the correlation between the electrophysiological inhomogenity of the mammalian ventricular moycardium in one heart and between different hearts of the same species and between the differences observed in the ion channel expression patterns.

Regarding the regional inhomogenity of the myocardium the transient outward potassium current (I_{to}) and the slow component of the delayed rectifier potassium current (I_{Ks}) had a good correlation to the underlying ion channel protein expressions. With electrophysiological methods we did not find differences in the parameters of the L-type

calcium current (I_{Ca}), the inward rectifier potassium current (I_{K1}) and the rapid component of the delayed rectifier potassium current (I_{Kr}). These data had a good correlation to the ion channel expression patterns as there were no differences in the protein levels in the examined regions of the myocardium. During the examination of the gender-related inhomogenity the found differences in the repolarization had a good correlation to the I_{to} and I_{K1} ion channel protein levels and these changes were accounted for the effects of testosterone.

The ventricular myocardium is not homogeneous as once it has been thought, indeed, many differences can be observed in the morphology of action potentials, in the amplitude and kinetics of the underlying ion currents, in distribution of the various ion channel proteins in the different regions and layers of the cardiac muscle and between different hearts of the same species. The physiological relevance of the transmural electrophysiological differences is the formation of various repolarization waveforms on ECG, including T and U waves that has great diagnostic significance. Another consequence of these transmural differences is the divergent responses of these cells to pharmacological agents, like antiarrhythmic drugs. It is straightforward that the more is known about these gradients and their changes during various pathological states, the more efficient and tailor-made therapy can be developed and applied in the pharmacological treatment of cardiac patients.

In extenso publications that provide the basis of the thesis:

1. **Szabó G**, Szentandrássy N, Bíró T, Tóth IB, Czifra G, Magyar J, Bányász T, Varró A, Kovács L, Nánási PP: Asymmetrical distribution of ion channels in canine and human left ventricular wall: epicardium versus midmyocardium. *Pflügers Arch* 2005;450:307-316 **[IF=3,564]**

2. Szentadrássy N, Bányász T, Bíró T, **Szabó G**, Tóth B, Magyar J, Lázár J, Varró A, Kovács L, Nánási PP: Apico-basal inhomogeneity in distribution of ion channels in canine and human ventricular myocardium. *Cardiovasc Res* 2005;65:851-860 **[IF=5,283]**

3. Fülöp L, Bányász T, **Szabó G**, Tóth IB, Bíró T, Lőrincz I, Balogh Á, Pető K, Mikó I, Nánási PP: Effects of sex hormones on ECG parameters and expression of cardiac ion channels in dogs. *Acta Physiol (Scand)* 2006;188:163-171 **[IF=2.23]**

Other in extenso publications:

4. Szabó A, Szentandrássy N, Birinyi P, Horváth B, **Szabó G**, Bányász T, Márton I, Nánási PP, Magyar J: Effects of articaine on action potential characteristics and the underlying ion currents in canine ventricular myocytes. *Br J Anaesthesia* 2007;99:726-733 **[IF=2.948]**

5. Szabó A, Szentandrássy N, Birinyi P, Horváth B, **Szabó G**, Bányász T, Márton I, Magyar J, Nánási PP: Effects of ropivacaine on action potential configuration and ion currents in isolated canine ventricular cardiomyocytes. *Anesthesiology* 2008 Apr;108(4):693-702; **[IF=4.596]**