Ph.D. THESIS

Role of potassium currents in the repolarization of canine left ventricular cardiomyocytes

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Introduction

I. General considerations

The plasmamembrane of the cells found in the human body separates spaces with different ion contets, the intra-, and extracellular field. The action potential (AP) of the excitable cells (so thus the ventricular cardiomyocytes) is in fact a voltage change caused by temporal and spatial alterations of ionic currents flowing through the cell membrane.

The membrane potential of a cell at a given moment determined by the electrochemical gradients of the different ions and the dissimilar permeability of the ion channels. This phenomenon is described by the Goldman–Hodgkin–Katz equation.

The electrochemical gradients are generated by several transport mechanisms, and these transports are also required to resettle the original ion distribution. These transports directly or indirectly utilize energy stored in ATP molecules. The Na^+/K^+ ATP-ase carries Na^+ ions from the cell to the extracellular space and in the meantime, it takes K^+ ions back into the cell from the extracellular space. The pump works with 3 Na^+/2 K^+ stochiometry, so it generates a small outward current. Another important ionic pump is the Na^+/Ca^{2+} antiport mechanism which transports 3 Na^+ and 1 Ca^{2+} ions at the same time so it is also electrogenic. It can result both in inward and in outward current (I_{NaCa}) depending on the momentary membrane potential and the ion distribution. Its function is indispensable for the Ca^{2+} homeostasis of the cells, it helps to eliminate Ca^{2+} got into the sarcoplasm during the AP.

The plasmamembrane is not equally permeable for the certain ions. At a given moment the membrane potential is the closest to the equilibrium potential of the mostly permeable ion.

At rest the plasmamembrane of the ventricular cardiomyocytes found in mammals is highly permeable for K^+ ions while the permeability for other (Na^+, Ca^{2+}, Cl^-) ions are close to zero. Consequently the resting membrane potential is close to the equilibrium potential of the K^+ ions. Under physiological

circumstances the inequal ion distribution, the permeability of the different types of ion channels and the function of electrogenic transports establish a resting membrane potential of approximately –80 mV, so at rest the ventricular cells are more negative than their environment.

The action potential itself is produced by the time-, and voltage dependent conductance changes of the ion channels found in the plasmamembrane (the conductance, in definition, is the reciproc of the ion channel's momentary resistance; it is proportional to the permeability of the given ion channel). Conventionally the AP is divided into five phases.

II. Action potential of the ventricular cardiomyocytes

II.1. Phase 0 and phase 1

Phase 0 is governed by the activation of time and voltage dependent fast Na^+ channels, thus multiplying the Na^+ conductance by many times. The electrochemical gradient for Na^+ is very large so the opening of Na^+ channels results in a huge current (I_{Na}). Accordingly the membrane potential shifts towards the Na^+ equilibrium potential so the membrane depolarizes. This phase is called the upstroke of the AP after the shape of the sudden and large membrane potential change observed on the voltage-time graph. The inactivation of the fast Na^+ channels rapidly occurs and they recover from inactivation only after the repolarization of the membrane.

The depolarization caused by the fast Na^+ channels activates some ion channels and closes or inactivates some others. The large K^+ current responsible for maintaining the resting membrane potential (inward rectifier K^+ current, I_{K1}) diminishes because of its inward rectification and this prevents the development of a repolarizing current during phase 0. In the meantime the transient outward current (I_{to}) is activated by the depolarization. I_{to} has K^+ and Cl^- ions as charge carriers. I_{to} is responsible for the early repolarization (phase 1) of the action potential.

II.2. Phase 2

An important factor for the genesis of the phase 2 (also called plateau phase) of the ventricular action potential is the high membrane resistance during this phase, which is mainly due to the inward rectification of I_{K1}. Inward and outward currents flowing during the plateau phase are in the same order of magnitude so the membrane potential changes relatively slowly.

The main channel providing inward current during the plateau phase is the L-type Ca^{2+} current. This current is activated more slowly than I_{Na}, but it also lasts longer. The gradual rise in the dalayed K^+ currents balances the depolarizing effect of the $I_{Ca,L}$.

It must be noted that there are marked differences in the shape of the action potentials recorded in different regions of the myocardium. In the subepicardial cells the phase 1 repolarization is very prominent, while it is very slight in subendocardial cells. The early repolarization of the midmyocardium is somewhere between the two formerly mentioned ones. These differences are most probably because of the different densities of the I_{to}.

II.3. Phase 3 and the blocade of I_K

The final repolarization (phase 3) which follows the plateau of the action potential, is largely initiated by the inactivation of the L-type Ca^{2+} current and by the activation of the delayed rectifier K^+ current (I_K). Later, the terminal repolarization is governed by the I_{K1}, which becomes larger at membrane potentials more negative than –55 mV.

On the basis of kinetics, rectification, sensitivity to blockers and modulation by intracellular messengers, three delayed K^+ currents can be distinguished: the slow component of I_K (I_{Ks}), the rapid component of I_K (I_{Kr}) and the ultrarapid component of I_K (I_{Kur}). Both I_{Kr} and I_{Ks} can be found in atrial and ventricular myocytes, but I_{Kur} is only present in the atria.

The selective blockers of I_{Kr} such as E-4031, dofetilide or D-sotalol are methanesulfonanilide structure compounds, and they greatly lengthens the AP

duration. This effect is exploited in class III. antiarrhythmic drugs. However blockade of K^+ currents, especially I_{Kr} can be observed in a wide spectrum of other drugs as well, such as antihistamines (terfenadine, astemizol), anti-microbal agents (erythromycine, ketokonazole), drugs affecting the gastro-intestinal tract (cisaprid) or antipsychotics (haloperidol). The common properties of the above listed drugs are that they have at least one aromatic ring and that they all block I_{Kr} by entering into the ion channel pore and „obstruct" the channel.

Any substance which inhibits the repolarization of the ventricular myocytes legthens the AP duration, and therefore it can cause acquired long QT syndrome. Both acquired and inherited forms of long QT syndrome enhance the risk of syncope, certain types of arrhythmias (eg. torsade de pointes type ventricular tachycardia) and sudden cardiac death.

Delayed rectifiers are important for regulating the repolarization of the sino-atrial node and the atrio-ventricular node as well, so thus they determine the maximal diastolic potential and affect the rhythym generation of these regions. Nevertheless in the pacemaker tissue the components of I_K show markedly different activation and deactivation kinetics, so different forms of the ion channels can be presumed.

II.4. Phase 4

During the phase 4 of the AP (electrical diastole) the membrane potential of the extranodal myocytes stays in the resting value until the beginning of the next AP. This physe is determined by a large K^+ conductance resulting in I_{K1} current. I_{K1} is responsible for maintaining the resting membrane potential close to the equlibrium potential of K^+. However these two values are not exactly the same because of the electrogenic ion pumps (Na^+/K^+ pump and Na^+/Ca^{2+} antiport) and the small but not zero conductance of other ions. During the electrical diastole the Na^+/K^+ pump generates a small outward, the Na^+/Ca^{2+} antiport generates a small inward current while the ionic currents other than I_{K1} resulting in an inward background current. The transport mechanisms are

required for re-establish the original inequal distribution of ions after the period of the AP. All these factors together result in a resting membrane potential 5-10 mV more positive than the equlibrium potential of K^+ ions.

Objectives

In the present study we aimed to characterize the four major K^+ currents (I_{to}, I_{Kr}, I_{Ks} and I_{K1}) thought to be active during ventricular repolarization in canine left ventricular myocytes. For this reason, mostly action potential clamp method has been used which can be carried out very close to the physiological conditions. Beyond the description of canine ventricular K^+ currents their frequency-dependent behaviour was also examined to elucidate better their role in ventricular repolarization.

The significance of studying canine myocytes under action potential clamp conditions is given by the fact that ventricular ion currents in the human heart most resemble those found in canine myocardium. That is why the experiments executed in canine model can be extrapolated to human correlation with only minimal implication.

Materilas and methods

I. Isolation of single canine left ventricular myocytes

All of our in vitro experiments were carried out on enzimatically isolated canine left ventricular cardiomyocytes. Single myocytes were obtained from adult dogs bred for experimental purposes using the anterograde segment perfusion technique.

After the isolation procedure 30-60 % of the cells were rod shaped and showed clear striation when the external calcium was restored to 2.5 mM. The experiments were started two-three hours after the isolation. The cells were stored in Minimum Essential Medium Eagle solution (pH=7.4) at 14 °C until use.

II. Electrophysiological measurements

Myocardial cells were transferred to a thermoregulated chamber (1 ml volume) mounted on the stage of an inverted microscope and superfused with Tyrode solution (144 mM NaCl, 5.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES, 10 mM glükóz; pH=7.4) at 37 °C. The flow rate was 10 ml/min. Axoclamp 2B an Axoclamp 200B amplifiers (Axon Instruments, Union City, CA, USA) were used in current clamp or voltage clamp mode. Digidata 1200 A/D-D/A converter operated under pClamp 6.0 software (Axon Instruments) was used to collect data and to deliver the command voltage.

II.1. Recording of action potentials from single canine myocytes

Transmembrane potentials were recorded at 37°C using sharp glass microelectrodes filled with 3 M KCl and having tip resistance between 20 and 40 MΩ. The cells were paced through the recording electrode at steady cycle length of 1 s using 1 ms wide rectangular current pulses with 120% threshold amplitude. Action potentials were digitized at 100 kHz and stored for later analysis.

II.2. Voltage-clamp technique

Electrodes were prepared from borosilicate glass, having tip resistance of 1.5–2.5 MΩ when filled with pipette solution containing: K-aspartate 100, KCl 45, $MgCl_2$ 1, EGTA 10, K-ATP 3 and HEPES 5 mM. Careful suction was applied to help gigaseal formation and the subsequent disruption of the membrane patch. Ionic currents were normalized to cell capacitance, determined in each cell using short (10 ms) hyperpolarizing pulses from –10 mV to –20 mV. Average cell capacitance was 127±8 pF, and the series resistance was typically 4–8 MΩ before compensation (usually 50–80%) prior to voltage clamp.

II.2.1. Action potential clamp

Action potentials were recorded in current clamp mode through the patch pipette in Tyrode solution. The cells were continuously paced through the recording electrode usually at a steady stimulation frequency of 1 Hz (or alternatively, at 0.2, 0.5, or 1.66 Hz) using 1 ms wide rectangular current pulses with suprathreshold amplitude. The AP obtained this way was delivered to the same cell at the identical frequency as command voltage after switching the amplifier to voltage clamp mode. The current trace obtained under these conditions was a horizontal line positioned at the zero level except for the capacitive transient. I_{to}, I_{Kr} and I_{K1} were dissected by application of 1 mM 4-aminopyridine, 1 μM E-4031, and 50 μM $BaCl_2$, respectively, each superfused for 3 min. The profile of these ion currents was determined by subtracting the pre-drug curve from the post-drug one. This procedure resulted in current profiles having inverted polarity.

II.2.2. Measuring I_{Ks} with rectangular pulses

I_{Ks} was activated by 5-s-long depolarizations to +30 mV applied at frequency of 0.1 Hz. When studying voltage dependence of activation, voltages were varied between –20 mV and +50 mV. The amplitude of the tail current was measured upon repolarization to the holding potential of –40 mV.

III. Mathematical modeling

We used computer simulation to investigate the effects of plateau height and action potential duration on the kinetic properties of I_{Ks}. The numerical model, describing the voltage and time dependence of I_{Ks}, published by Viswanathan et al., was modified and used to predict the profile of the current during the action potential. Action potentials, recorded previously from canine ventricular myocytes predominantly of midmyocardial origin, were used to drive the model to compute the profile of I_{Ks}. To test the effect of the altered action potential duration or plateau height, we developed a computer program, which could set the plateau height or action potential duration of the recorded action potential to the required values. Thus, a series of action potentials having differences only in the studied parameter were generated from a single action potential.

Mathcad 2000i (MathSoft Engineering & Education, Surrey, UK) was used to run the model. A 10-ms period of lag at the resting potential was applied before the action potential to reach steady state conditions for the current before activation. The integration time step was 1 ms. Because the model involves changes of the cytosolic calcium concentration, an artificially constructed calcium transient was inserted into the model. The diastolic and systolic calcium concentrations were set to 200 nM and 2 μM, respectively.

IV. Statistics

All values presented are arithmetic means±SEM. When comparing the groups, one-way ANOVA, Student's t-test for paired and unpaired data was used as appropriate. Correlations between two variables were determined using linear regression. Differences were considered significant when the P value was less than 0.05.

Results

I. K^+ currents under action potential clamp conditions

I.1. Transient outward current (I_{to})

I_{to} was dissected by application of 1 mM 4-aminopyridine, which is known to block 70 % of I_{to} with little effect on other ventricular K^+ currents. At a constant stimulation frequency of 1 Hz, I_{to} rose rapidly upon depolarization, peaked at 4.4±0.7 ms after the action potential upstroke, and decayed to zero with a time constant of 7.4±0.6 ms. As a consequence, the 4-aminopyridine-sensitive current fully disappeared by the 50th ms of the plateau (0.05±0.1 pA/pF in the average of 10 cells). The peak amplitude and the integral of I_{to} were 3.0±0.23 pA/pF and 29.7±2.5 fC/pF, respectively. The time of I_{to} peak always preceded the deepest point of the initial repolarization of action potential. According to the current-voltage relationship, generated by plotting Ito values against their respective membrane potentials, the current was active between −15 and +40 mV, and its maximum was reached at +8.8±1.9 mV. The membrane potential at the deepest point of the early repolarization was inversely related to the peak value of I_{to}. No correlation was found between action potential duration, measured at 90 % of repolarization (APD_{90}), and the peak value of I_{to}.

I.2. Rapid delayed rectifier K^+ current (I_{Kr})

I_{Kr} was studied in nine myocytes at 1 Hz using 1 µM E-4031 to block the current selectively. I_{Kr} gradually increased during the plateau to give a peak value of 0.62±0.08 pA/pF and an integral of 57.6±6.7 fC/pF. The maximum of I_{Kr} was measured at −54.2±1.7 mV. Good correlation was found between the time of I_{Kr} peak and the time of the maximal velocity of repolarization (V^-_{max}), actually I_{Kr} peaked 7 ms before the V^-_{max}.

When I_{Kr} was compared with the net membrane current (I_{net}), estimated as the product of the membrane capacitance and the first time-derivative of the

action potential ($I_{net}=-C_m*dU/dt$), I_{net} was always greater than I_{Kr} during the plateau. At the time of V^-_{max}, I_{Kr} was only 25 % of I_{net}, indicating that a current other than I_{Kr} is dominant during the terminal repolarization. Congruent with this, no correlation was found between neither the amplitude of I_{Kr} and V^-_{max}, nor between I_{Kr} and APD_{90}.

I.3. Slow delayed rectifier K^+ current (I_{Ks})

Current profile of I_{Ks} tried to be shown in the presence of 10 μM chromanol-293B at 1 Hz in five myocytes. The amplitude of the chromanol-sensitive currtent did not exceed the triple of the noise level, that is why we finally decided not to describe the I_{Ks} under action potential clamp conditions.

I.4. Inward rectifier K^+ current (I_{K1})

I_{K1} was visualized in seven cells at 1 Hz by applying 50 μM $BaCl_2$. I_{K1} was non-zero during diastole: its amplitude was 0.26±0.03 pA/pF. The current transiently decreased after the upstroke, then increased slowly during the plateau. As determined from the I-V relation, the rise of I_{K1} accelerated at –22 mV, and its peak amplitude of 1.8±0.1 pA/pF was reached 1 ms after the time of V^-_{max} at –58.3±0.6 mV. The integral of I_{K1} was 61.6±6.2 fC/pF. This could be divided into two components: a charge of approximately 40 fC/pF was associated with the current transient flowing during terminal repolarization, and almost 20 fC/pF was carried during the plateau. I_{K1} represented 75 % of net membrane current at the time of V^-_{max}. Accordingly, good correlation was obtained between the maximum velocity of repolarization and the peak amplitude of I_{K1}. Similarly in the results obtained with I_{Kr}, no correlation was found between I_{K1} and APD_{90}.

I.5. Frequency-dependent properties of I_{Kr} and I_{K1}

As frequency-dependent changes in action potential configuration are based on the frequency dependence of the individual ion currents involved in ventricular repolarization, I_{Kr} and I_{K1} were studied also at lower (0.2 Hz) and higher (1.66 Hz) frequencies. Neither the peak value, nor the mid-plateau

value of I_{Kr} was dependent on the stimulation frequency. Similarly, the amplitude of I_{K1} was also independent of the rate of pacing, and the maximum velocity of repolarization was also uniform within the studied range of frequency.

We measured, therefore, the dependence of I_{Kr} tail current amplitude on the proximity of the preceding depolarization under conventional voltage clamp conditions using a twin-pulse protocol. Pairs of depolarizations to +10 mV (250 ms in duration) were applied from the holding potential of –80 mV, and tail currents appearing at –40 mV after repolarization of the second pulse were registered. These experiments were performed in the presence of 5 lm nifedipine and 30 μM chromanol-293B to eliminate $I_{Ca,L}$ and I_{Ks}, respectively. The parameters of the applied pulses were chosen to mimic a pair of action potentials separated by gradually increasing interpulse intervals. According to the results the proximity of the pulses (varied from 25 ms to 10 s) had no influence on the tail current amplitude, indicating that accumulation of I_{Kr} fails to occur.

Furthermore the relation of the membrane current determined at the middle portion of the plateau phase and APD_{90} was described. The net membrane current can be calculated from the membrane capacitance and the rate of voltage change. These experiments were performed in current clamp mode to record action potentials at frequencies ranging from 0.2 to 2.5 Hz. Our results proved that APD_{90} was inversely related to the net membrane current flowing at the middle portion of the plateau.

II. Investigation of I_{Ks} with chromanol-293B

II.1. Effect of chromanol on action potentials

Chromanol caused a frequency dependent lengthening of action potentials without changing their other parameters. APD50 and APD90 were increased by 10 μM chromanol from 152.0±7.4 to 158.0±10.9 ms and from 200.5±8.2 to 209.7±10.8 ms, respectively, at the cycle length of 1 s. This effect, corresponding to 6.0±1.6 and 9.2±3.5 ms lengthening of APD50 and APD90,

respectively, was moderate but statistically significant and increased with increasing the cycle length. No changes regarding other parameters of the action potential could be observed in the presence of chromanol.

II.2. Effect of chromanol in the presence of isoproterenol and E-4031

The effect of 10 μM chromanol on action potential duration was measured after pretreatment with 2 nM isoproterenol and in the presence of 1 μM E-4031. Isoproterenol increased the amplitude of the plateau with no significant effect on APD_{95}. E-4031 increased both APD_{95} and the amplitude of plateau. These effects were statistically significant at all cycle lengths longer than 0.5 s, and were enhanced at longer cycle lengths. The chromanol-induced lengthening of APD was significantly augmented by the presence of isoproterenol, as well as by E-4031, in a reverse rate-dependent manner.

One of the main goals of this study was to identify the determinant parameter(s) of the action potential responsible for activation of I_{Ks}, which was assessed from the magnitude of the chromanol-induced lengthening of APD. Therefore, the lengthening effect of chromanol was correlated with the amplitude of the plateau and with APD_{95}, both measured in the presence of isoproterenol or E-4031. The lengthening effect of chromanol showed good correlation with the amplitude of plateau in the presence of isoproterenol and E-4031, whereas no correlation was found with APD_{95} in isoproterenol and E-4031. These results indicate that the amplitude of the plateau is an important determinant of the activation of I_{Ks} during the action potential independently of the way of plateau elevation.

II.3. Effect of chromanol in myocytes injected with inward current pulses

Because isoproterenol was shown to alter the kinetics of I_{Ks} in canine cardiac preparations, a strategy allowing to study the effect of plateau elevation without altering the phosphorylation state of the $K_v7.1$ channel responsible for I_{Ks} current. Therefore we applied rectangular inward current pulses in current clamp mode. The beginning of these pulses was

synchronized to action potential upstroke, their duration was set to 170 ms, and their amplitude was increased up to 500 pA in 100 pA steps. The series of current injection was repeated before and after chromanol treatment in the same cell allowing to asses the lengthening effect of chromanol in a relatively wide range of plateau amplitude and APD_{95} values. The current pulses increased both the amplitude of plateau and duration of action potentials. These effects were greater in the presence than in the absence of 10 μM chromanol. Similarly, to results obtained with isoproterenol and E-4031, the chromanol-induced lengthening was highly proportional to the amplitude of plateau, while no correlation was found with the duration of the action potential.

II.4. Simulation of the profile of I_{Ks} during the action potential

Using the modified model of Viswanathan et al. the effect of the plateau height and action potential duration on the density of I_{Ks} was studied. Series of action potentials, generated from a single action potential recorded previously so as they differed from one another only in their plateau height or APD_{95}, were used to drive the model to compute the profile of I_{Ks}. Values of plateau height and APD_{95} were chosen to cover the range obtained with isoproterenol, E-4031, and current injection. The peak amplitude of the computed I_{Ks} was a sharp function of the amplitude of plateau growing up close to fourfold when the plateau height was increased from 72 to 107 mV. IKs was proportional also to APD_{95}; however, almost doubling APD_{95} (changing it from 180 to 350 ms) increased the maximum of I_{Ks} by less than 50 %.

Our results suggest that it is mainly the amplitude of the plateau phase which mostly influences the activation of I_{Ks}.

III. Az L-364,373 hatása az I_{Ks}-re kutya bal kamrai szívizomsejteken

Because of the I_{Ks} can not be described under action potential clamp conditions, we aimed to enhance this current via a pharmacological way. For this purpose, L-364,373 compound seemed to be an ideal candidate. This

compound was described as a potent I_{Ks} activator; it enhanced I_{Ks} in micromolar concentrations in rabbit and guinea pig preparations. Before the application of L-364,373 during action potential clamp conditions we had to firstly prove that it has the same I_{Ks} enhancing effect in our canine ventricular myocytes. For these experiments we used conventional voltage-clamp method. As a reference, mefenamic acid has been used.

100 μM mefenamic acid significantly increased the amplitude of the fully activated I_{Ks} measured at +30 mV (2,.8±0.47 pA/pF vs. 4.46±0.98 pA/pF), as well as I_{Ks} tail current amplitudes recorded upon returning to the holding potential of –40 mV (1,04±0,14 pA/pF vs. 1,77±0,39 pA/pF). The action of mefenamic acid was voltage dependent: augmentation of I_{Ks} was more pronounced at less positive voltages. The I_{Ks}-stimulating effect of mefenamic acid was due to a reversible negative shift on the voltage dependence of activation. In addition, mefenamic acid increased the time constant obtained for deactivation of I_{Ks} (74±15 ms vs. 186±37 ms).

The effect of L-364,373 was tested at concentrations of 0.1, 1 and 3 μM (n=5, 6 and 6, respectively). L-364,373 — in contrast to mefenamic acid — had no enhancing effect on the amplitude of I_{Ks}, at any membrane potential studied. In fact, both the fully activated I_{Ks} (3,94±0,36 pA/pF; and 3,62±0,53 pA/pF; 3,59±0,53 pA/pF; 3,08±0,46 pA/pF in normal Tyrode solution and in the presence of 0,1 μM; 1 μM and 3 μM L-364,373 respectively) and I_{Ks} current tails (1,23±0,1 pA/pF, and 1,21±0,13 pA/pF; 1,15±0,12 pA/pF; 1,14±0,04 pA/pF, respectively) were moderately diminished by 3 μM L-364,373. Similarly to mefenamic acid, L-364,373 increased the time constant of deactivation (66±3 ms, and 73±8 ms; 78±8 ms és 87±10 ms, respectively).

Discussion

The exact role of the certain K^+ currents in canine left ventricular myocytes has not been cleared yet. The goal of our experiments were to clarify the exact role of four potassium currents (I_{to}, I_{Kr}, I_{Ks}, I_{K1}) found in canine left ventricular myocardium. The profile of three of these currents (I_{to}, I_{Kr}, I_{K1}) could be obtained under action potential clamp conditions. This method is the most convenient to study the ionic currents closest to physiological conditions, allowing the description of each current during an action potential. Beyond the description of canine ventricular K^+ currents their frequency-dependent behaviour was also examined to elucidate better their role in ventricular repolarization.

I. K^+ currents under action potential clamp conditions

In the present work we visualized the true profile of three important K+ currents (I_{to}, I_{Kr} and I_{K1}) thought to be involved in repolarization of canine ventricular myocytes, and gave parametric description of these currents. As two of them (I_{Kr} and I_{K1}) show strong inward going rectification, their profile can hardly be studied under conventional voltage clamp conditions based on application of rectangular voltage pulses.

The profile of I_{Ks} can not be described using action potential clamp therefore we tried to deduce the role of I_{Ks} by applying its specific blocker.

The 0.62 pA/pF peak amplitude of I_{Kr} obtained at 1 Hz with action potential clamp is comparable with the 0.55 pA/pF value reported previously in dog, but is lower than the 0.86 pA/pF found in guinea pigs myocytes. Similarly, the 1.8 pA/pF peak I_{K1} measured in our canine myocytes is less than that reported by others in guinea pigs cells. Taken into account that $I_{Ca,L}$ is also more pronounced during the action potential plateau in guinea pigs than in dogs, it appears that canine myocytes work more economically than guinea pig cells because they allow less inward as well as outward currents to flow while maintaining the electrical balance during their plateau.

In our experiments both I_{Kr} and I_{K1} were building up gradually as the membrane became more and more repolarized during the phase of terminal repolarization, however, I_{Kr} peaked 8 ms earlier and at 4 mV less negative membrane potential than I_{K1} resulting in further acceleration of repolarization, and consequently, in the 'ignition' of I_{K1}. Indeed, the very narrow membrane potential ranges where I_{Kr} and I_{K1} peaked (–54.2±1.7 and –58.3±0.6 mV, respectively) suggest that 'activation' of these currents during repolarization is governed by voltage rather than time. In line with this concept of positive feed-back mechanism of terminal repolarization, the contribution of peak I_{Kr} and peak I_{K1} to the net membrane current, measured at the time of V_max, was roughly 1:3. The good correlation found between peak I_{K1} amplitude and V^{-}_{max} indicates that the velocity of terminal repolarization is primarily determined by the magnitude of I_{K1} in canine cells.

On the other hand, no correlation was found between action potential duration and the peak amplitude of any of the currents studied suggesting that the timing of repolarization depends on the fine balance of all inward and outward currents active during the plateau. Although the net charge carried by I_{to}, I_{Kr} and I_{K1} was in the same order of magnitude (29.7, 57.6 and 61.6 fC/pF, respectively), their role in controlling action potential duration seems to be markedly different. Because of its fast decay time constant (7.4 ms), I_{to} rapidly and fully disappeared during the early plateau allowing little contribution as a charge carrier to the later phase of repolarization. Indeed, Sun and Wang elegantly demonstrated in their recent dynamic clamp experiments that 'insertion' of Ito into endocardial canine action potentials produced the characteristic spike-and-dome morphology, but failed to modify action potential duration. Based on the results above, the contribution of Ito to repolarization was not analysed further in the present work.

The slow component of the delayed rectifier K^+ current can not be visualized properly due to technical difficulties. The amplitude of the chromanol-sensitive current was very small, that is why we finally decided not to describe the I_{Ks} under action potential clamp conditions.

Focusing to I_{Kr} and I_{K1} we found that Inet was close to the sum of I_{Kr} plus I_{K1} at the middle portion of the plateau at 1 Hz. This does not necessarily mean that IKr and IK1 are the only currents active during the mid-plateau, it rather implicates that the other inward and outward currents flowing during the plateau may well be balanced by each other.

Action potential clamp experiments are suitable for visualization of true ion current profiles, provided that a specific blocker of the current was applied and full block was achieved. These requirements were perfectly met with E-4031, the specific blocker of I_{Kr}. Unfortunately, neither 4-aminopyridine nor $BaCl_2$ are specific inhibitors in concentrations high enough to fully block I_{to} or I_{K1}, respectively. The best strategy in such a case is to use lower concentrations (believed to be sufficiently specific) – as it was done in the present study. $BaCl_2$ (50 µM) was shown to block 88 % of I_{K1} at –58 mV, where the current peaked during repolarization. The I_{to} blocker 4-aminopyridine was used in 1 mM concentration, which was able to block approximately 70% of I_{to}. Thus correction factors of 1.14 and 1.43 have to be applied when estimating the true values of I_{K1} and I_{to}, respectively. 4-aminopyridine (1 mM) seems to suppress I_{to} quite selectively, as no hump was detected on the terminal phase of the 4-aminopyridine-sensitive current trace, when both I_{K1} and I_{Kr} have their maximal amplitude during terminal repolarization.

Rate-dependent properties of action potential duration have been studied since long time and interpreted in terms of the frequency dependence of the underlying ion currents. Our results indicate that neither I_{Kr} nor I_{K1} is frequency-dependent in dog ventricular cells within the studied frequency range of 0.2–1.66 Hz. This is not surprising in the case of I_{K1}, but – because of its slow deactivation kinetics – accumulation of I_{Kr} was claimed to contribute to the well-known rate-dependent shortening of action potential duration observed at faster stimulation frequencies. In contrast to its slow deactivation, I_{Kr} cannot accumulate, as the amplitude of I_{Kr} was independent of the proximity of the preceding depolarization. This, in turn, implicates that $K_v11.1$ channels (responsible for I_{Kr}) which remain open after terminal repolarization of the

action potential must rapidly close during the subsequent action potential upstroke allowing no accumulation of I_{Kr} to occur.

These results are in line with previous observations obtained in guinea-pig ventricular myocytes and HEK cells expressing HERG channels suggesting that I_{Kr} is not frequency-dependent within a wide range of stimulation frequencies. In endocardial canine myocytes Hua & Gilmour also failed to demonstrate significant ratedependent changes in I_{Kr} at frequencies lower than 2 Hz; however, in that study a reduction in peak I_{Kr} and an increase in diastolic I_{Kr} was observed at frequencies higher than 3.3 Hz.

I_{K1} tail currents were also shown to be modified at high frequencies, although the changes were completely relaxed within 0.3 s. These results suggest that the mechanisms responsible for ratedependent changes in action potential duration at fast and slow heart rates may also be different. In summary, our results indicate that neither I_{Kr} nor I_{K1} is frequency-dependent in canine ventricular cells up to 1.66 Hz under our experimental conditions. Consequently, these currents – similar to I_{to} and I_{Ks} – may have limited chance to induce frequency-dependent changes in action potential duration at normal or low heart rates.

II. Contribution of I_{Ks} to repolarization

Based on the results obtained from studying the APD lengthening effect of chromanol in canine ventricular myocytes two important statements can be made. Firstly, we have demonstrated that IKs does contribute to normal ventricular repolarization because chromanol caused a moderate but statistically significant lengthening of APD in this species at cycle lengths longer than 0.5 s. Secondly, we have shown that contribution of IKs to repolarization is primarily determined by the plateau height. The augmentation of the chromanol induced lengthening of APD observed in the presence of isoproterenol or E-4031 confirms the results of previous studies.

Regarding the first statement, it must be noted that those investigators who concluded that I_{Ks} failed to contribute to repolarization in canine myocytes

under baseline conditions have also observed some APD lengthening with chromanol, as well as with other selective I_{Ks} blockers, like HMR-1556, or L-735,821. In our experiments, however, these differences were statistically significant, although the magnitude of lengthening was moderate. On the other hand, the lengthening effect of chromanol was dramatically increased by all interventions resulting in the elevation of action potential plateau (pretreatment with isoproterenol, E-4031, or application of inward current). These results were corroborated by computer simulations indicating that elevation of the plateau amplitude within the range of 72–107 mV (corresponding to –8 to +27 mV of plateau potential considering an approximately –80 mV resting potential) caused an almost fourfold increase in the maximal amplitude of I_{Ks}. The sharp increase in I_{Ks} amplitude observed in response to plateau elevation is clearly due to the increased voltage-dependent activation of IKs and may account for the apparent discrepancies in the literature. For instance, in guinea pig ventricular cells the plateau potential is well above +30 mV, in contrast with canine Purkinje fibers having their plateau potentials in the negative voltage range. Accordingly, prominent APD lengthening was observed in the former preparation, while practically none in the latter one in response to I_{Ks} blockade. In our experiments, the plateau potential was around +10 mV, which is congruent with a moderate effect of chromanol at baseline. Nakashima et al. came to the same conclusion reporting delayed ventricular repolarization in response to inhibition of I_{Ks} in the presence of beta-adrenoceptor blockade.

In contrast to the good correlation found between the chromanol-induced APD lengthening and the plateau height, virtually no correlation was observed between the lengthening effect of chromanol and APD_{95}. This seems to be in conflict with the linear relationship demonstrated by the simulation study.

The contradiction can be resolved considering that in the presence of E-4031, and also in the case of current injection, both APD_{95} and the plateau amplitude were simultaneously increased. In contrast, during our in silico studies we changed only one parameter of the action potential at a time. If the in silico results are true, the correlation between the APD_{95} and the amplitude

of I_{Ks} will be much more complicated to prove in vitro because of the small steepness of the relation. This means that the APD_{95} and the amplitude of I_{Ks} might be correlated but we could not prove this in vitro because of the random fluctuation in the amlitude of plateau.

Varro et al. observed strong enhancement of the chromanol-induced APD lengthening in canine ventricular myocardium, when the action potential was prolonged by E-4031 or veratrine, suggesting that APD is an important determinant of the chromanol-induced lengthening. Because both drugs are known to increase the plateau height substantially, contribution of this latter effect to the observed lengthening cannot be ruled out. It must be mentioned, however, that the largest chromanol-induced lengthening in our experiments was obtained in the presence of E-4031 — in spite of the less pronounced elevation of the plateau induced by E-4031 comparing to isoproterenol. This result could have been interpreted as an evidence supporting the role of action potential duration. Considering the flat APD_{95}-lengthening relationship, it seems to be more likely that suppression of I_{Kr} increased the relative contribution of I_{Ks} to repolarization, and this might cause the increased lengthening effect of chromanol. The same argumentation may be used to explain the reverse rate dependent nature of the chromanol-induced lengthening. I_{Kr} is much stronger at shorter than at longer cycle lengths due to its frequency dependent accumulation diminishing the relative significance of I_{Ks} at fast heart rates. Such accumulation of I_{Ks} was not observed in canine myocytes at cycle length longer than 500 ms. Based on the speculation above one may predict an enhanced lengthening effect of chromanol under all circumstances when the repolarization reserve is compromised.

Because configuration of the cardiac action potential critically depends on the fine balance between several inward and outward currents, all manipulations leading to elevation of the plateau will necessarily modify all the ion currents contributing to ventricular repolarization. For instance, in addition to enhancement of the two delayed rectifier K+ currents (I_{Kr} and I_{Ks}) the driving force for Ca^{2+} entry through the L-type Ca^{2+} channel will also be diminished by

elevation of the plateau potential resulting in a smaller density of inward current, with the concomitant outward shift in the net membrane current, which, in turn, will increase the repolarization reserve. The opposite can be anticipated in the presence of isoproterenol, which is known to increase Ca^{2+} current several fold. Of course, modification of the other currents has also to be considered, which may distort the evaluation of the role of I_{Ks} in repolarization.

II.1. Clinical implications

The original concept for development of I_{Ks} blocker drugs, such as chromanol, as potential antiarrhythmic agents was based on the assumption that they would be less proarrhytmic comparing to I_{Kr} blockers because their effect was anticipated to be less affected by the disadvantageous reverse rate dependent mode of action, a common characteristic of pure I_{Kr} blockers. Several studies — including this one — suggest, however, that this is not fully the case. In this study, the lengthening effect of chromanol was tested in two situations occurring most typically under pathological conditions. Low concentration (2 nM) of isoproterenol was used to mimic the effect of strongly increased sympathetic tone, which is associated with several cardiac disorders including heart attack. The results indicate that the APD lengthening effect of chromanol (the desired class 3 antiarrhytmic action) was markedly enhanced in the presence of isoproterenol. This suggests — in spite of the limited contribution of I_{Ks} to normal repolarization — that a substantial APD lengthening effect can be expected *in vivo*, especially when the sympathetic tone is elevated. Similar conclusions were drawn by Volders et al. and Jost et al. when studying canine and human ventricular myocardium. One might argue that suppression of I_{Ks} in this situation may potentially be beneficial because itmay counterbalance the shortening of APD evoked by the sympathetic activation resulting in prolongation of the refractory period. Although I_{Ks} blockade was shown to decrease dispersion of repolarization in failing canine hearts, marked heterogeneities in action potential duration with resultant

arrhythmias were observed when the I_{Ks} blockade was applied after stimulation of beta-adrenoceptors. The I_{Kr} blocker E-4031 was applied to simulate the compromised repolarization reserve, which can be either inherited or acquired. This latter form, very often caused by noncardiac medication, sometimes remains hidden. In these patients the already compromised repolarization reserve would further be reduced by the I_{Ks} blockade, increasing the risk of torsades de pointes type of arrhythmias. If the I_{Ks} blockade is introduced at low heart rate or increased sympathetic activity combined with partially suppressed I_{Kr}, the risk of fatal outcome may be even higher. These data suggest that I_{Ks} blockade may carry a significant risk of proarrhythmia. In conclusion, the strategy of IKs blockade as a potential therapeutic intervention seems to be rather harmful than beneficial, however, conclusive clinical investigations are required to clarify the therapeutic value of I_{Ks} blockers.

III. L-364,373 fails to enhance I_{Ks} in canine ventricular cardiomyocytes

Previously it was shown that the I_{Ks} could not be described under action potential clamp conditions mainly due to its very small amlitude. That is why we examined whether we can amplify this current with az I_{Ks} activator compound (L-364,373), so that we can carry out the measurements with action potential clamp technique.

The major finding of this study is that the benzodiazepine derivative L-364,373, up to the concentration of 3 μM, failed to enhance I_{Ks} in canine ventricular myocytes. This is in sharp contrast to previous results obtained in guinea pig and rabbit myocytes, where 0.1–1 μM L-364,373 markedly increased I_{Ks} due to causing a leftward shift in its activation curve. Consequently we could not use the L-364,373 compound for our action potential clamp measurements.

The molecular background of the lack of I_{Ks}-enhancing effect of L-364,373 in canine myocardium is not clear at present. Mefenamic acid was shown to activate I_{Ks} only if its pore-forming α subunit (K_v7.1, KCNQ1) was coexpressed with its β subunit (MinK, KCNE1).. In contrast to these observations, L-364,373

was ineffective when the channels were formed by coassembly of K_v7.1 and MinK subunits. Although both mefenamic acid and L-364,373 are supposed to bind to the Kv7.1 subunit, the two distinct binding sites are allosterically modified by the association of MinK in an opposite way. Therefore, interspecies differences in the MinK/K_v7.1 ratio may be a possible candidate to explain our results. Comparison of the efficacy of L-364,373 in guinea pig and rabbit is highly congruent with this explanation. Salata et al. observed a fourfold increase in IKs in the presence of 0.1 µM L-364,373 in guinea pig, and the maximal effect, which was a more than 15-fold (!) increase in I_{Ks}, was achieved at 1 µM. In contrast to this, 1 µM L-364,373 caused a much weaker effect in rabbit, and lower concentrations were not tested at all in this species.

The alternative possibility that canine I_{Ks} channels fail to bind L-364,373 can easily be ruled out, since both compounds (L-364,373 and mefenamic acid) exerted qualitatively similar effects on the deactivation time constant of canine I_{Ks}.

In summary, it can be concluded that studies performed in human cardiomyocytes are required to evaluate the effects of I_{Ks} activators. Based on the present results the potential therapeutical effect of L-364,373 in human myocardium became questionable. This is to be strengthened in further studies, however, it is now clear that rodent cardiac tissues are not suitable for this purpose.

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