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Profile of the main ionic currents of cardiac action potential and their calcium dependence

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Introduction

Cardiovascular diseases, including ventricular arrhythmias, still has a leading role in European mortality rates. Despite the improvement of clinical electrophysiology and cardiology there are many cases, when we cannot prevent the development of arrhythmias and sudden cardiac death. Therefore, in order to treat the most frequent, often fatal diseases, it is essential to know the physiological and pathophysiological background of the diseases as much as possible.

The configuration of the cardiac action potential (AP) depends on the finely tuned balance of sequentially activating inward and outward currents. The current density at any moment is determined by the driving force acting on the ion and the conductance of the channel, influenced by its time- and voltage-dependent gating kinetics. This latter is precisely described by the conventional voltage clamp methodology, where kinetic properties of ion currents are studied using rectangular voltage protocols. However, during the action potential the membrane potential is continuously changing resulting in a concomitant alteration of the driving force. This problem was resolved by the introduction of the action potential voltage clamp technique, which is based essentially on pharmacological current dissection using the original action potential waveform of the cell as a command signal. The action potential voltage clamp technique was successfully applied in a variety of mammalian cardiac cells, including rat, pigs, rabbits, guinea pigs, dogs and humans.

Despite the abundance of action potential voltage clamp data, little attempt was made to study the correlation of various ion current densities within the same cell. In order to obtain multiple ion current data from one single myocyte, the “onion peeling” technique has been developed. This method allows recording of several ion current profiles from the same myocyte under largely physiological conditions by applying sequential pharmacological

dissection. However, apart from two studies performed in rabbit, porcine and guinea pig myocytes such data are not available in the literature.

Under pathological conditions, the regulation and expression pattern of ion channels may change, and the various drugs may reduce or enhance their function, causing pro-arrhythmic state. These may affect the function of multiple organ systems in addition to the cardiovascular system.

The correlations among the ion current parameters are less known, however, in order to adequately treat arrhythmias, it is essential to know the current profile of each ion conductance of the action potential and the correlations among these ion currents. Exploring these relationships may open a new chapter in the treatment of arrhythmias.

Aims

Numerous studies have examined the ionic currents of cardiomyocytes, however, these results are contradictory, and the experiments were performed under conditions far from physiological (room temperature, different ionic composition). Therefore, in this study, our objectives were:

• to investigate the relationship between the different ionic currents involved in the formation of action potential under action-potential voltage-clamp conditions.

• to study the correlations between the parameters of depolarizing and repolarizing currents involved in action potential formation.

• to investigate the events of action potential terminal repolarization under physiological conditions, as well as the calcium dependence of repolarizing potassium currents (I_{K1}, I_{Kr}, I_{Ks}) and late sodium current.

Materials and methods

Electrophysiology

The experiments were performed on enzymatically isolated canine left ventricular cardiomyocytes. Single canine myocytes were obtained by enzymatic dispersion using the segment perfusion technique. All animal handling and laboratory procedures conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and to our Institutional Animal Care and Use Committee approved protocols.

Transmembrane ionic currents were registered using the whole-cell configuration of the patch-clamp technique and action potential voltage-clamp (APVC) technique. Briefly, the action potential voltage clamp technique is a special electrophysiological method in which the cell is stimulated with a pre-registered action potential instead of a square pulse. To avoid the consequences of cell-to-cell variations in action potential morphology, measurements were performed using a so called “canonic” action potential as the command signal. This canonic action potential was chosen as a representative midmyocardial canine action potential characterized by average parameters. Application of uniform command action potentials makes the comparison of the individual current traces easier. The current trace obtained under these conditions was a horizontal line positioned at the zero level except for the capacitive transient. This is considered the reference current. Using the specific ion channel inhibitor the inhibited ion current must be compensated by the amplifier. Subtracting the compensation current from the reference current yields the drug-sensitive ion current profile under the action potential.

The onion peeling technique is based on consecutive pharmacological dissections using selective ion channel inhibitors. During these measurements the sequence of drug-application is critically important. First the inward currents were dissected in the following sequence: $I_{Na,L} \rightarrow I_{NCX} \rightarrow I_{Ca,L}$. This was

followed by the dissection of outward currents in the same cell with the sequence of: $I_{Kr} \rightarrow I_{Ks} \rightarrow I_{K1}$. The advantage of the onion peeling technique is that it is possible to study multiple ion currents on the same myocardial cell.

The ionic currents were defined as an inhibitor-sensitive current obtained by pharmacological subtraction. Ion currents were normalized to cell capacitance. The dissected currents were evaluated by determining their maximal densities (peak currents), their densities measured at the half-duration of the command action potential (mid-plateau current densities), and the total charge carried by the current (current integrals).

Conventional voltage-clamp experiments, using rectangular command pulses were also performed, to examine the L-type calcium current. During the action potential measurements, the action potentials were recorded in current-clamp mode with sharp microelectrodes. All the experiments were performed at 37 ^{o}C.

Intracellular Ca^{2+} concentration and cell shortening measurement

Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)was visualized by FURA-2-AM fluorescent dye. The intracellular Ca^{2+} transients were recorded using an alternating dual beam excitation fluorescence photometry setup. Excitation wavelengths of 340 and 380 nm were used to monitor the fluorescence signals of Ca^{2+}-bound and Ca^{2+}-free Fura-2 dye, respectively, while fluorescent emission was detected at 510 nm. The ratio of F_{340}/F_{380} fluorescence intensity is proportional to the intracellular Ca^{2+} concentration.

Single cell shortening was recorded by optical edge detection. Cells were field stimulated using suprathreshold square wave pulses. Cell shortening was measured using a video edge detector system. Cell shortening was expressed as a percent of initial diastolic cell length. These experiments were carried out at 37 $^{\circ}$C.

Statistics

Results are expressed as mean ± SEM values, n denotes the number of myocytes studied. Statistical significance of differences was evaluated using ANOVA followed by Student's t-test. Correlations between two variables were determined using correlation analysis. Correlation was characterized by the Pearson correlation coefficient (r) and the slope (m). Significance of the correlation coefficient (p_r) and slope (p_m) was determined using t-test. Differences and correlations were considered significant when p values were less than 0,05.

Results

Current profiles, densities and correlations

In the case of inward currents, the largest one was $I_{Ca,L}$, I_{NCX} was intermediate and $I_{Na,L}$ was the smallest when compared on the basis of current integrals. The current integrals of $I_{Ca,L}$, I_{NCX} and $I_{Na,L}$ were -72.2±6.0 mC/F; -37.3±2.5 mC/F and -27.7±2.3 mC/F respectively. Comparing outward currents in the same way, I_{K1} was the largest (64.9±3.9 mC/F), I_{Kr} was intermediate (34.1±3.9 mC/F) and I_{Ks} was the smallest (15.6±1.3 mC/F). When the comparison was made on the basis of mid-plateau current density, the largest inward current was still $I_{Ca,L}$ (-0.44±0.03 A/F), but the largest outward current was I_{Kr} (0.13±0.02 A/F), while the smallest one was I_{K1} (0.06±0.01 A/F).

Correlations between individual ion currents were studied. No correlation was found between the integrals of the individual outward currents. In contrast, good correlation was observed between the integral of $I_{Ca,L}$ and I_{NCX}, but none between those of other inward currents. Furthermore, no correlation was found between inward or outward currents when compared at peak or mid-plateau

levels. Comparison of inward and outward currents yielded good correlation only between $I_{Ca,L}$ and I_{Kr}, importantly, this correlation was significant for both peak current density and current integral. In case of mid-plateau current density the correlation was not significant statistically between $I_{Ca,L}$ and I_{Kr}. Not too surprisingly, significant correlation was observed between the summed integrals of inward and outward currents as well.

Events of terminal repolarization

The profiles and time course of outward currents involved in action potential terminal repolarization were examined and the maximal rate of repolarization (V^{-}_{max}) of action potential was calculated. The latter parameter corresponds to the net current flowing through the membrane (I_{net}) during terminal repolarization, which is defined as the first derivative of the value of the membrane potential. Although the peak density of I_{Kr} was smaller than that of I_{K1}, I_{Kr} peaked earlier during the action potential, at a less negative membrane potential than I_{K1}. More importantly, I_{Kr} peaked 11 ms before, while I_{K1} peaked 3.5ms after the time of V^{-}_{max} suggesting that I_{Kr} may be dominantly responsible for the timing of action potential duration in canine ventricular myocytes.

The calcium dependence of outward currents during the repolarization were also examined. Since the sequence of ion current dissection was started with inward currents, all outward currents were measured in the presence of inhibitors of inward currents (1 μM GS-967 + 0.5 μM ORM-10962 + 1 μM nisoldipine). Because of the blockade of L-type calcium channels by nisoldipine, both the submembrane and the bulk cytosolic [Ca^{2+}] was likely reduced in these myocytes compared to the situation of free Ca^{2+} cycling. Therefore, to see how normal Ca^{2+} cycling and $I_{Ca,L}$ blockade affect the outward currents of interest, a set of further experiments were performed when only the outward currents were dissected with undisturbed intracellular Ca^{2+} homeostasis.

It was found that I_{Ks} profiles, and consequently their phase-plane trajectories, were markedly different depending on $[Ca^{2+}]_i$. Comparing to the case of free Ca^{2+} cycling, peak current density (0.20 ± 0.02 *versus* 0.32 ± 0.06 A/F), mid-plateau current density (0.08 ± 0.02 versus 0.23 ± 0.03 A/F) and current integral (15.6 ± 1.4 versus 28.2 ± 5.2 mC/F) were significantly smaller, while I_{Ks} reached its peak significantly later (171 ± 5 versus 154 ± 4 ms) after $I_{Ca,L}$ blockade. In our experimental setting, only I_{Ks} was Ca^{2+}-sensitive. No significant differences were observed in peak current density, mid-plateau current density and current integral of I_{Kr} or I_{K1} when compared under conditions of normal Ca^{2+} cycling and following $I_{Ca,L}$ blockade.

The effect of ionic current inhibition on calcium transients of cardiomyocytes

To demonstrate the changes in $[Ca^{2+}]_i$ caused by the consecutive inhibition of $I_{Na,L}$, I_{NCX} and $I_{Ca,L}$, $[Ca^{2+}]_i$ was recorded in control, after superfusion with 1 μM GS-967 and finally with 1 μM GS-967 + 0.5 μM ORM-10962.

It was found that neither the block of $I_{Na,L}$ nor the block of I_{NCX} changed significantly the systolic and diastolic $[Ca^{2+}]_i$ values. However $[Ca^{2+}]_i$ transient amplitude (the difference between systolic and diastolic $[Ca^{2+}]_i$ values) was decreased significantly by GS-967 the blocker of $I_{Na,L}$, while no further change was observed after the cumulative addition of ORM-10962 (blocker of I_{NCX}). Since $[Ca^{2+}]_i$ measurements could not be performed with nisoldipine due to the high photosensitivity of the drug, the effect of nisoldipine was studied by measuring unloaded cell shortening. The systolic, but not diastolic, cell length increased significantly and the fractional shortening decreased to a value close to zero by 1 μM nisoldipine, suggesting a robust reduction in the amplitude of $[Ca^{2+}]_i$ transient following the inhibition of inward currents.

The calcium-dependence of the late sodium current

The $I_{Na,L}$ was examined under action potential voltage clamp conditions, 1 μM GS967 and 10 μM TTX dissected similar inward current profiles in canine ventricular cells. In cardiac myocytes, both agents selectively inhibit Na^+ currents. The densities, measured at 50% of APD_{90} (-0.42±0.03 versus -0.40±0.04 A/F) and integrals (-68.0±5 versus -61.0±6 mC/F) of the dissected currents were largely comparable in size (APD_{90} is the action potential duration measured at 90% repolarization). Ca^{2+}-sensitivity of $I_{Na,L}$ was studied by blocking $I_{Ca,L}$ with 1 μM nisoldipine in order to reduce the Ca^{2+} entry into the myocytes. $I_{Na,L}$ was smaller following nisoldipine pretreatment than under control conditions. In case of GS967, the current densities, measured at 50% of APD_{90}, and current integrals were significantly lower in the presence than in the absence of nisoldipine. Similar results were found if $I_{Na,L}$ was dissected with 10 μM TTX, however, the difference in current integrals was not significant in this case. There are two possible explanations for this behavior. First, GS967 might also suppresses $I_{Ca,L}$ at the applied concentration. Therefore, when GS967 is used without L-type calcium channel blockade, the GS967-sensitive current will be contaminated with a small fraction of $I_{Ca,L}$. Second, $I_{Na,L}$ could be modulated by changes of intracellular Ca^{2+} concentration. In this case, $I_{Na,L}$ becomes smaller, as $[Ca^{2+}]_i$ is reduced by nisoldipine pretreatment. To differentiate between these options, the effect of GS967 was studied on $I_{Ca,L}$ under conventional voltage clamp conditions. Neither peak $I_{Ca,L}$, nor its density measured at 50 ms after the beginning of the pulse, was altered by 5 min superfusion with 1 μM GS967. Similarly, no change was observed in the current integral measured before and after application of GS967. This result excludes the contamination of the GS967-sensitive current ($I_{Na,L}$) with $I_{Ca,L}$, and supports the selective action of GS967 on $I_{Na,L}$. Therefore, the results can be interpreted as the reduction of $I_{Na,L}$ in the presence of nisoldipine is related to the $[Ca^{2+}]_i$-dependent behavior of $I_{Na,L}$, as reported previously in rabbit myocytes.

To support the role of $[Ca^{2+}]_i$ in the regulation of $I_{Na,L}$ the cytosolic Ca^{2+} was reduced by 10 mM BAPTA added to the pipette solution. In these experiments, measurements started 10 min after rupturing the seal to let the Ca^{2+}-chelator equilibrate between the pipette solution and the intracellular space. In the presence of intracellular BAPTA the $I_{Na,L}$ current density, measured at 50% of APD_{90} (-0.30±0.03 A/F), and the current integral (-46.7±5.2 mC/F) were significantly lower than obtained under control conditions (-0.42±0.03 A/F and -68.0±5 mC/F, respectively).

The Ca^{2+}-dependent behavior of the GS967-sensitive current could be demonstrated also under current clamp conditions when action potentials were elicited by electrical stimulation. 1 μM GS967 caused significant shortening of action potential duration (measured at 90% repolarization), in a reverse rate-dependent manner and decreased significantly the amplitude of plateau (defined as a difference between the plateau potential measured at 50% of APD_{90} and the resting membrane potential). The GS967-induced plateau depression was strongly reduced when the cells were pretreated with 5 μM BAPTA-AM for 30 min, while the magnitude of the APD shortening effect of GS967 remained similar after loading the cells with the Ca^{2+}-chelator. The effect of BAPTA-AM was strongly dependent on the pacing cycle length: APD was significantly prolonged by BAPTA-AM at longer cycle lengths, while no significant change was caused at shorter ones. These results indicate that the GS967-induced plateau-depression, which can be related to the reduction of $I_{Na,L}$, is $[Ca^{2+}]_i$-dependent, since it appeared only in cells with normal calcium homeostasis. Unexpectedly, the APD shortening effect of GS967 was similar with or without BAPTA-AM pretreatment. This can probably be ascribed to the BAPTA-AM-induced lengthening of APD developing at longer cycle lengths only. Since the effect of many cardioactive drugs on APD is proportional to the baseline value of APD, this might compensate for the expected reduction of the GS967-induced APD shortening after reduction of $[Ca^{2+}]_i$ by BAPTA-AM pretreatment.

The role of CaMKII in regulation of $I_{Na,L}$

To test the hypothesis of Ca^{2+}-dependent augmentation of $I_{Na,L}$, the role of CaMKII, the most likely candidate to mediate Ca^{2+}-dependent changes, was studied. In these experiments the pipette solution contained 1 μM KN-93, the inhibitor of CaMKII, and the measurements were started after 10 min equilibration with KN-93. The results indicate that the current density measured at 50% of APD_{90} was significant smaller with KN-93 than under control conditions. The current integral was also smaller with KN-93, however, this change did not reach the level of statistical significance. Upon comparing our results obtained with KN-93, nisoldipine and BAPTA, neither the current densities at 50% of APD_{90} (-0.28±0.04, -0.29±0.04 and -0.30±0.03 A/F, respectively), nor the respective current integrals (-54.6±9.9, -48.2±3.6 and -46.7±5.2 mC/F) were significantly different between these groups. These results suggest that in the presence of nisoldipine and BAPTA, a reduced CaMKII activity could possibly cause a significantly smaller $I_{Na,L}$ compared to the normal intracellular calcium homeostasis condition.

To test whether the concentration of free calmodulin in the cell has any influence on $I_{Na,L}$, the current was also measured using pipette solutions containing 100 nM calmodulin. In these experiments $I_{Na,L}$ was determined as a 10 μM TTX-sensitive current and recordings started 10 min after the rupture of the gigaseal. No significant differences were observed in either the current densities at 50% of APD_{90} or the current integrals respectively, in the presence and absence of calmodulin.

Discussion

Current profiles, densities and correlations among the parameters of ion currents

This is the first study to investigate the correlation between the various ion currents in canine ventricular myocytes using the onion peeling technique. Also, we are the first to report the Ca^{2+}-sensitivity of I_{Ks} and INa,L in this species. No correlation was observed between the inward or outward currents, except for the positive correlation of $I_{Ca,L}$ and I_{NCX}. This is not surprising bearing in mind that the major pathway for Ca^{2+} entry is the L-type Ca^{2+} channel, while the dominant mechanism of Ca^{2+} removal from the cell is the Na^+/Ca^{2+} exchanger in canine cardiac myocytes – similarly to other mammalian species.

One might expect some correlation not only between $I_{Ca,L}$ and I_{NCX} but also between $I_{Na,L}$ and I_{NCX}, since changes in Na^+ entry alter I_{NCX} function by changing its equilibrium potential and the Na^+ load. While the correlation between $I_{Ca,L}$ and I_{NCX} integrals were significant , the correlation coefficient between $I_{Na,L}$ and I_{NCX} integrals was only 0.42, which is not significant statistically. Considering that largely the same amount of Ca^{2+} is extruded from the myocyte by the forward mode I_{NCX} activity as enters through L-type calcium channels, $I_{Ca,L}$ and I_{NCX} are very closely connected in regulating $[Ca^{2+}]_i$, hence a strong correlation is expected. Regarding the Na^+ homeostasis of a healthy, paced cardiomyocyte, Na^+ channels are responsible for only about 22% of the total Na^+ entry (approximately half of which is via $I_{Na,L}$), while forward mode I_{NCX} accounts for 60% of the total sodium entry. This suggests that the Na^+ homeostasis of a healthy cell is mainly governed by the amount of Ca^{2+} entry, and the consequent Ca^{2+} removal though I_{NCX}. Furthermore, changes in the intracellular Na^+ homeostasis can be compensated by other Na^+-coupled transport mechanisms, like Na^+/K^+ pump, Na^+/H^+ exchanger, or the Na^+/HCO^-_3

cotransporter. Therefore, a strong correlation between $I_{Na,L}$ and I_{NCX} is not expected.

I_{Kr} was the only outward current showing good correlation with $I_{Ca,L}$, the largest inward current. Even though the peak current density and integral of I_{Kr} were not the largest among outward currents, IKr was the dominant outward current in the middle of the plateau phase. The correlation between $I_{Ca,L}$ and I_{Kr} can be caused by proportional gene expression, common folding or trafficking of ion channel proteins mediating these currents, or a connection in regulatory pathways. Even though the exact nature of these mechanisms needs further clarification, such correlation between two major inward and outward currents in the middle of the plateau phase may help to maintain the stability of the ventricular action potential duration by minimizing its heterogeneity.

It is not exceptional that a dominant outward current is expressed proportionally with a dominant inward current, since in guinea pig $I_{Ca,L}$ correlates with I_{Ks} instead of I_{Kr}. In guinea pig the dominant repolarizing current during the plateau is I_{Ks} - in contrast to canine ventricular cells. In other preparations, like porcine and rabbit myocytes, strong positive correlation was demonstrated between $I_{Na,L}$ and I_{Kr}. This is in contrast to our results in dog, where no significant correlation was seen between any parameters of these two currents. Indeed, it appears that $I_{Na,L}$ counterbalances I_{Kr} in rabbit during the action potential plateau, which does not seem to be the case in canine myocytes. These results suggest that large interspecies differences may exist regarding the correlation between the relative density of various ion currents, however, the major question, i.e. the correlation pattern of the individual inward and outward currents in human ventricular myocardium remains unanswered.

The profiles and timing of ionic currents during terminal repolarization of action potential

The timing of events of terminal repolarization points out the significance of I_{Kr} in repolarization of canine ventricular action potential. During phase 2 repolarization (as the net membrane current is turned to be more and more outward due to the progressive decay of inward and rise of outward currents) I_{Kr} peaked earlier, while I_{K1} peaked later than the time of V^{-}_{max}, furthermore, I_{Kr} peak developed at less negative membrane potential than I_{K1} peak. Therefore - although both I_{Kr} and I_{K1} share the property of inward going rectification – it is more likely that I_{Kr}, but not I_{K1}, is the initiator of terminal repolarization in spite of the fact that the peak value of I_{K1} is much greater than the peak of I_{Kr} at this critical period of time. Further arguments supporting this interpretation comes from pharmacological studies showing that the selective I_{Kr} inhibitor E-4031 or dofetilide causes a pronounced lengthening of action potential at the level of APD_{50}, while the I_{K1} inhibitor Ba^{2+} causes rather triangulation of the action potential due to prolongation of APD_{90} dominantly with a relatively moderate effect on APD_{50}. It must be mentioned, however, that the properties of I_{Kr} and I_{K1} are independent of action potential morphology or the pacing frequency in canine and human ventricular myocytes. This is important to see because any distortion of the action potential-ion current relationship, resulting potentially from the application of canonic instead of self action potentials, may not modify the temporal relationship observed between I_{Kr} and I_{K1}, which is a crucial point of our argumentation.

Our results show that I_{Ks} peak appears earlier during the action potential than that of I_{Kr}. It deserves some speculation, especially because compared to I_{Kr} the slow component of the current (I_{Ks}) activates at more positive voltages and its activation time constant is more than one order of magnitude longer compared to I_{Kr}. Upon depolarization, I_{Kr} activates rapidly, which is followed by

a practically immediate inactivation, making I_{Kr} almost zero in the first tens of milliseconds of the canine action potential. I_{Kr} starts to functionally occur during the following part of repolarization because the channels start to recover from inactivation. This happens only after the membrane potential has achieved a sufficiently negative value. This rapid and pronounced inactivation of I_{Kr} limits the number of conducting channels in the early phase of the plateau. Indeed, inactivation properties of I_{Kr} critically determined the duration of action potentials in a previous dynamic clamp study. On the other hand APD was little influenced by the selective I_{Ks} blocker L-735,821 in canine ventricular cells under baseline conditions. These findings also confirm the pivotal role of I_{Kr} in timing of terminal repolarization.

Calcium sensitivity of ionic currents

Another important result of this work was to show that out of the three studied outward currents only I_{Ks} was Ca^{2+}-dependent in canine ventricular cells under close to physiological circumstances, using the action potential voltage clamp technique. I_{Ks} accumulated faster, its amplitude and integral were greater under conditions of free Ca^{2+}-cycling than after $I_{Ca,L}$ blockade. The underlying mechanism of Ca^{2+}-sensitivity of canine I_{Ks} is complex, however, similar results were obtained by others in rabbit ventricular cells, where the Ca^{2+}-sensitivity of I_{Ks} was linked to the activity of calmodulin, while a calmodulin – PIP2 competition was reported in human Kv7.1 channels expressed in CHO cells.

The Ca^{2+}-dependent nature of I_{Ks} may help to dissolve the discrepancy between previous results obtained in canine myocytes under action potential voltage clamp conditions where the maximal density of I_{Ks} was in the range of 0.10–0.15 A/F– corresponding to our results obtained following $I_{Ca,L}$ blockade, which were less than half of the value measured under conditions of free Ca^{2+}-cycling. In those experiments the intracellular medium contained Ca^{2+}-chelators in the millimolar range – in contrast to the present experiments. In contrast to

some earlier results, I_{K1} was not influenced by the $I_{Ca,L}$ blockade in the present study. Reduction of $[Ca^{2+}]_i$ augmented I_{K1} in guinea pig myocytes, an effect explained by the reduction of inward going rectification of I_{K1} caused by the lower cytosolic Ca^{2+}. In contrast, elevation of $[Ca^{2+}]_i$ has also resulted in an increased I_{K1} in canine ventricular cells, an effect attributed to the Ca^{2+}-dependent activation of CaMKII, and the concomitant augmentation of I_{K1}. It must be noted that in the study of Nagy et al., $[Ca^{2+}]_i$ was permanently clamped at different levels with EGTA, whereas no calcium buffers were used in the measurements presented here. The lack of difference in I_{K1} under conditions of normal Ca^{2+}-cycling and after $I_{Ca,L}$ blockade in the current study may be explained by the relatively low baseline activity of CaMKII in canine myocytes. In the results of Nagy et al., a permanent elevation of $[Ca^{2+}]_i$ to around 900 nM increased the amplitude of I_{K1}. At this continuously high $[Ca^{2+}]_i$ value the activation of CaMKII is definitely expected – in contrast to the much lower normal $[Ca^{2+}]_i$ of the current study. It is also possible, however, that in our study, stimulation of I_{K1} by CaMKII activation during the normal calcium transients and the suppressive effect of the Ca^{2+}-dependent inward going rectification effectively blunted each other.

This is the first time to investigate the modulation of $I_{Na,L}$ by changes in $[Ca^{2+}]_i$ in canine ventricular cells under physiological conditions, since reduction of $[Ca^{2+}]_i$ by application of 10 mM intracellular BAPTA or by superfusing the cell with 1 μM nisoldipine significantly decreased both the density and the integral of $I_{Na,L}$. The Ca^{2+}-dependency of the $I_{Na,L}$ was evident under both action potential voltage clamp and current clamp conditions. Furthermore, this Ca^{2+}-dependent modulation of $I_{Na,L}$ was due to the contribution of CaMKII in regulation of the current, because inhibition of CaMKII with KN-93 decreased the density of the current to the level observed with nisoldipine or BAPTA. The CaMKII-related stimulation of $I_{Na,L}$ observed by us in healthy canine ventricular

cells was similar to those reported by in rabbit, murine, canine and human myocytes under various pathological conditions, like heart failure, cardiac hypertrophy, ischemia or hypoxia.

Present results also indicate that in paced canine ventricular cardiomyocytes with intact calcium homeostasis (just like in the physiologically active, working ventricle) CaMKII is partially activated. This is indicated by the marked reduction of $I_{Na,L}$ in response to decreasing $[Ca^{2+}]_i$ or inhibition of CaMKII. This is important because in a previous work, performed at room temperature in canine myocytes, $I_{Na,L}$ was compared only between zero (chelated with EGTA or BAPTA) and high (1 μM) levels of $[Ca^{2+}]_i$. A limitation of the present work is that $I_{Na,L}$ was not studied under conditions when CaMKII is maximally activated at high $[Ca^{2+}]_i$ levels, so the total CaMKII-dependent fraction of $I_{Na,L}$ could not be estimated.

While it is generally believed that KN-93 binds directly to CaMKII, and thus preventing kinase activation by competing with Ca^{2+}/CaM, recent data suggest that KN-93 binds directly to Ca^{2+}/CaM and not to CaMKII. Although the mode of action presented by Wong et al. is consistent with KN-93 being regarded as a functional CaMKII inhibitor, the ubiquity of Ca^{2+}/CaM regulation imposes the question whether the KN-93-based observations (like data presented in our study) could partly or fully be explained by a Ca^{2+}/CaM-dependent, but CaMKII-independent inhibition. It has been shown that Ca^{2+} itself may regulate sodium channels, while others suggest that it is the Ca^{2+}/CaM complex that regulates voltage-gated sodium channels. There is a general consensus, however, that the steady-state inactivation curve of Na^+ channels is shifted toward more positive voltages by higher $[Ca^{2+}]_i$. Nevertheless, even a small shift in the steady-state inactivation curve might be enough to create a larger $I_{Na,L}$ in cardiac cells with normal calcium homeostasis, compared to the conditions when nisoldipine, BAPTA, or KN-93 is applied.

An additional message of the present work is related to the fact that blockers of L-type Ca^{2+} channel (like nisoldipine) are often used to separate drug actions targeting cardiac L-type Ca^{2+} current. It has to be born in mind, however, that these blockers do not only switch off the L-type Ca^{2+} current by itself, but as a result, $[Ca^{2+}]_i$ is also decreased. Therefore, L-type Ca^{2+} channel blockers potentially interfere with all Ca^{2+}-dependent processes, such as CaMKII activity. In turn, reduction of baseline CaMKII activity may cause the concomitant reduction of many Ca^{2+}-dependent ion currents, including $I_{Na,L}$.

New scientific results

1. This is the first time to report the strong correlation between the amount of charge delivered by $I_{Ca,L}$ and I_{NCX}, the peak- and the plateu density, and the amount of charge delivered by $I_{Ca,L}$ and I_{Kr} on canine left ventricular cardiomyocytes.

2. We demonstrated the strong calcium-dependence of the slow component of the delayed rectifier potassium current (I_{Ks}) on canine left ventricular cardiomyocytes.

3. Strong calcium dependence was also observed in the case of the late sodium current ($I_{Na,L}$), which may be due to changes in the activity of the CaMKII pathway.

4. We found that in canine ventricular myocardial cells, during terminal repolarization, I_{Kr} reached its maximum 11 ms earlier than V^{-}_{max}, while I_{K1} reached its maximum 3.5 ms later. Based on these results, we hypothesize that under physiological conditions, I_{Kr} may be responsible for initiating terminal repolarization, thus determining the length of action potential.

Summary

The electric activity of cardiomyocytes is shaped by the concert of ion channels. If this tightly regulated system breaks down for some reason, it can cause severe conditions such as arrhythmias. The former studies about the ion channels of the cardiomyocytes were mostly performed on rodent models using conventional electrophysiological techniques. In addition, these measurements were often performed under conditions other than the physiological environment (room temperature, the presence of Ca^{2+}-chelators).

The aim of our scientific work was to investigate the profiles of the ion currents of ventricular myocardial cells during the ventricular action potential, under physiological conditions. We also investigated the correlations among parameters of these ionic currents. We performed experiments to investigate the calcium dependence of the ionic currents as well. Canine ventricular cardiomyocytes were used, because their electrophysiological properties under the action potential are similar to those of human ones.

The profile of the ionic currents were determined by action potential voltage clamp technique. The so-called onion peeling technique were used to obtain multiple ion current data from the same cell. Action potential measurements were used to examine the changes of action potential duration. Changes in the calcium cycle were confirmed by measuring calcium transient and cell length shortening.

We found a significant correlation between amplitude and mid-plateau density of fast component of the delayed rectifier potassium current (I_{Kr}) and the L-type calcium current ($I_{Ca,L}$). There were significant correlation between the carried charge by the I_{Kr} and $I_{Ca,L}$, and by the sodium-calcium exchange current (I_{NCX}) and the L-type calcium current ($I_{Ca,L}$). We found that the slow component of the delayed rectifier potassium current (I_{Ks}) and the late sodium current ($I_{Na,L}$) shows a strong calcium dependence. In the case of $I_{Na,L}$, the role of the CaMKII pathway was confirmed in the background of the calcium dependence.

Our results emphasize the complexity of the electrical functioning of the heart. Even more accurate knowledge about these currents may bring us closer to understand the pathophysiology of arrhythmias and to find a suitable therapeutic target for the treatment of various arrhythmic diseases.

Keywords

carciac cellular electrophyiology, late sodium current, I_{Ks}, I_{Kr}, I_{K1}, NCX, L-type calcium current, cardiomyocyte, APVC, CaMKII, ventricular arrhytmias

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List of publications related to the dissertation

1. Horváth, B.*, **Kiss, D. Z.***, Dienes, C., Hézső, T., Kovács, Z., Szentandrássy, N., Almássy, J., Magyar, J., Bányász, T., Nánási, P. P.: Ion current profiles in canine ventricular myocytes obtained by the "onion peeling" technique.
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* These authors contributed equally this work.
IF: 5 (2020)

2. **Kiss, D. Z.***, Horváth, B.*, Hézső, T., Dienes, C., Kovács, Z., Topal, L., Szentandrássy, N., Almássy, J., Prorok, J., Virág, L., Bányász, T., Varró, A., Nánási, P. P., Magyar, J.: Late Na+ Current Is [Ca2+]i-Dependent in Canine Ventricular Myocytes.
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List of other publications

3. Dienes, C., Hézső, T., **Kiss, D. Z.**, Baranyai, D., Kovács, Z. M., Szabó, L., Magyar, J., Bányász, T., Nánási, P. P., Horváth, B., Gönczi, M., Szentandrássy, N.: Electrophysiological Effects of the Transient Receptor Potential Melastatin 4 Channel Inhibitor (4-Chloro-2-(2-chlorophenoxy)acetamido) Benzoic Acid (CBA) in Canine Left Ventricular Cardiomyocytes.
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   Front. Pharmacol. 11, Article 413, 2020.
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