Calcium-dependent modulation of the cardiac repolarization

PhD Thesis

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STUDIES RELATED TO THE THESIS

I. *Norbert Nagy, Viktória Szűts, Zoltán Horváth, György Seprényi, Attila S. Farkas, Károly Acsai, János Prorok, Miklós Bitay, Attila Kun, János Pataricza, Julius Gy. Papp, Péter P. Nánási, András Varró, András Tóth*
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INTRODUCTION

Principles of generation of the cardiac AP

The electric integrity of the heart is an essential requirement for its synchronized activity and its adaptation to various external conditions. A crucial point of the beat-to-beat regulation of the heart is the fine-tuning of the APD, achieved primarily by a strict control of the repolarizing K^+ currents. In the past decades and also recently, a number of studies provided evidence that physiological regulation of the repolarization process, as well as the corresponding ionic currents, is a complex mechanism and may be substantially modulated by Ca^{2+} signaling pathways. Considering that the Ca^{2+} cycle of the heart is also subject to large variations under different conditions (i.e. frequency changes, adrenergic modulation, a number of severe cardiac diseases) a dynamic interrelationship should exist between intracellular Ca^{2+} handling and AP, which may substantially influence the normal cardiac adaptation or in certain conditions may lead to arrhytmia propensity. Therefore, a detailed analysis of this relationship may significantly improve our currently limited understanding on the mechanisms of physiological and pathological alterations of cardiac repolarization, as well as substantially facilitates the development of new pharmacological strategies.

Underlying ionic currents of the AP

The cardiac AP reflects coordinated activity of multiple ion channels that open, close and/or inactivate with different kinetics. Upon activation cardiomyocytes are depolarized by a rapid inflow of Na^+ ions generating a large and fast inward Na^+ current (I_{Na}). I_{Na} not only defines the rapid upstroke of the AP (phase 0) but has also primary role in defining the velocity of impulse propagation through the heart. The initial depolarization of the AP is followed by a transient repolarization (phase 1) mainly governed by the transient outward current (I_{to}). In this section of the AP the Ca^{2+} activated Cl^- current ($I_{Cl(Ca)}$ or I_{to2}) is also suggested to contribute to the transient repolarization. The rapid upstroke of the AP activates the L-type Ca^{2+} current (I_{CaL}) and also several crucial K^+ currents involved in the repolarization: i.e. the rapid and slow components (I_{Kr} and I_{Ks}) of the delayed rectifier K^+ current and the inward rectifier K^+ current (I_{K1}). During a period of time I_{CaL} opposes the outward repolarizing currents, establishing a nearly isoelectric plateau phase (phase 2) of the AP. The duration of the plateau phase has important role in controlling the amount of the Ca^{2+} influx. The Ca^{2+} influx has crucial role in initializing the intracellular Ca^{2+} cycle leading to contraction of the cell. When Ca^{2+} channels begin to close the outward currents gradually overcame I_{CaL}, enabling fast repolarization of the AP (phase 3), governed by cooperative function of I_{Ks}, I_{Kr}, and I_{K1}. The terminal repolarization (phase 4) of the AP, as well as the resting membrane potential is primarily controlled by the I_{K1}. Several

electrogenic ionic pumps and exchangers may also substantially contribute to shaping of the AP. The electrogenic Na^+/K^+ ATPase (NKE) has a pivotal role in setting the uneven distribution of Na^+ and K^+ ions between the intra- and extracellular spaces, thus enabling the excitability of the cardiomyocytes. The Na^+/Ca^{2+} exchanger (NCX), which is also electrogenic, may also contribute to the AP. Since the equilibrium potential of the NCX lies in the range of AP, both inward and outward currents can be generated. However the exact role of the NCX during an AP is not yet fully elucidated.

Significance of the cardiac repolarization reserve

The concept and terminology of the *repolarization reserve* was introduced by Roden in 1998, based merely on clinical observations. The principle of the repolarization reserve is a redundancy of the repolarization process, i.e. loss of one repolarizing current may not lead to excessive AP lengthening, since other unimpaired K^+ channels may provide sufficient repolarizing capacity. Therefore, it has critical importance in stabilizing the APD, refractoriness and conduction of the electric impulses. Furthermore, it restricts excessive AP lengthening caused by impaired channel function, e.g. in LQT's, extreme bradycardia, hypokalaemia, hypotiroidism, diabetes mellitus, drug exposure, etc. The repolarization reserve has also important role in decreasing the transmural dispersion of the repolarization, thus preventing cardiac arrhythmias. The key players of the reserve are I_{Kr}, I_{Ks}, I_{K1}, and presumably I_{to}. These repolarizing currents play a critical role in normal myocardial function, furthermore, the majority of life-threatening arrhythmias are known to derive from repolarization abnormalities. Therefore, better understanding of the pathomechanisms leading to AP abnormalities, and the development of novel strategies to treat arrhythmias require much more detailed knowledge of the repolarization mechanisms, including all its determinants (e.g.: $[Ca^{2+}]_i$).

Intracellular Ca^{2+} homeostasis of the heart

The purpose of the excitation-contraction (ECC) coupling of the heart is to provide effective and in the same time adaptive cardiac output by activation of the myofilaments. The ECC is governed by a highly complex mechanism initiated by depolarization of the sarcolemma and finalized by the contraction-relaxation cycle. Ca^{2+} has a central role in ECC: the primary point is the *Ca^{2+} influx* through the L-type Ca^{2+} channels which open by depolarization. The Ca^{2+}-influx triggers a substantially larger *Ca^{2+} release* from the sarcoplasmic reticulum (SR) which is called Ca^{2+} induced Ca^{2+} release. This transient $[Ca^{2+}]_i$ increase (*Ca^{2+} transient, CaT*) is terminated by both Ca^{2+} *reuptake to the SR* (via the activity of the SR Ca^{2+} pump, SERCA2a), and Ca^{2+} *extrusion (efflux) from the cell* (primarily via the forward mode activity of the NCX).

Since cardiac myocytes consist of several intracellular compartments, the rise in $[Ca^{2+}]_i$ is highly inhomogeneous. From this point three important compartments can be distinguished: (i) the *dyadic space* (fuzzy space), located between the terminal cistern of the SR and inner side of the sarcolemma (ii) the *submembrane area*, a narrow region under the sarcolemma, (iii) and the *bulk cytosol* which is the largest compartment of the intracellular space, located around the contractile proteins. It is important to note that during the CaT the $[Ca^{2+}]_i$ in the dyadic space reaches considerably larger magnitude and declines much faster compared to that in the bulk cytosol. Consequently the ion channels and transporters are located in the plasma membrane, are subject to markedly larger (and faster) beat-to-beat Ca^{2+} fluctuations, than can be measured in the cytosol by the conventional fluorometric technique.

Cardiac Ca^{2+} handling is tightly regulated by both intrinsic and extrinsic mechanisms. Intrinsic regulation is the *autoregulation of* $[Ca^{2+}]_i$, achieved by a simple negative and/or positive feed-back of $[Ca^{2+}]_i$ on Ca^{2+} influx and efflux, respectively. This mechanism always sets stable $[Ca^{2+}]_i$ level by fine tuning the Ca^{2+} content of the SR *via* balancing the Ca^{2+} influx and efflux at the same time. The extrinsic regulating mechanism is achieved by the neurohormonal system, which adjusts the activity of heart to meet the requirements of the body in various physiological or pathological situations (e.g. physical activity, stress, pregnancy, or several diseases etc.).

Interactions between AP repolarization and Ca^{2+} handling

Cardiac contraction is achieved by large elevation in $[Ca^{2+}]_i$, therefore sarcolemmal K^+ channels sense the beat-to-beat fluctuations in Ca^{2+} levels. The magnitude of the CaT shows large variability even in physiological conditions (during exercise, or emotional stress due to the adrenergic activation), furthermore in a variety of heart diseases (e.g.: heart failure, ischaemia/reperfusion injury, long-QT syndrome), where Ca^{2+} levels are also considerably influenced (increased or decreased). Therefore, it seems feasible that subsarcolemmal Ca^{2+} levels are able to modulate cardiac K^+ currents, thus could provide a further major mechanism to improve AP adaptation under various conditions.

Theoretically, $[Ca^{2+}]_i$-dependent modulation of a K^+ ion channel can be established either directly or indirectly, via the Ca^{2+}-dependent calmodulin (CaM), or the calmodulin-dependent protein kinase II (CaMKII). Nonetheless, the entire mechanism is probably more complicated, since shifts in Ca^{2+} levels are often the consequence of the activation of adrenergic pathways raising PKA and/or PKC. Since PKA and PKC may exert various effects (activation or inhibition) on K^+ currents, one may speculate that the actual effect on a given K^+ channel might be the result of multiple, simultaneously activated controlling pathways. In theory, the regulation of the repolarization process by Ca^{2+} signaling may have important role considering the following points:

i. Under normal conditions and heart rates, a K^+ channel may be dynamically modulated by alterations in subsarcolemmal Ca^{2+}, thus contributing to fine tuning repolarization and APD.

ii. In large mammals, APD and $[Ca^{2+}]_i$ show parallel changes upon frequency adaptation, therefore the increase or decrease in $[Ca^{2+}]_i$ may substantially promote the prompt frequency adaptation of APD

iii. $[Ca^{2+}]_i$ mismanagement, frequently observed in cardiac diseases may significantly influence K^+ channel function.

Aims of the study

The primary goal of the present study was to elucidate a few aspects of the possible modulatory effect of changes in $[Ca^{2+}]_i$ on repolarization and to elucidate its functional consequences.

- **First, we investigated the possible physiological role of the small conductance Ca^{2+} activated K^+ channels (SK-channels) in the repolarization process in rat, canine and human myocardium,**
- **Second, we attempted to elucidate the effect of $[Ca^{2+}]_i$ rise on I_{K1}. The possible consequences of this relationship on the cardiac repolarization reserve were also investigated.**

RESULTS

Functional analysis of the cardiac SK channels

Expression of SK2 channel protein in canine and rat ventricular myocardium

The SK2 channel protein was abundantly expressed in both canine and rat ventricular tissues. Results of the semiquantitative assay revealed approximately similar levels of SK2 protein expression in both species. To exclude the contribution of SK2 proteins derived from vessels, Western-blot analysis was also performed in isolated rat and canine ventricular cells. SK2 protein was clearly detectable at 60 kDa in both species, with similar protein levels.

For further confirmation, the distribution of SK2 channel protein was detected directly in isolated myocytes. Confocal microscopic images of immunostained cardiomyocytes also revealed comparable surface distribution of SK2 channel protein in both canine and rat ventricular cells.

Measurements of intracellular CaTs

Since both the investigation of SK-channels and I_{K1} requires the presence of CaTs in the cardiac preparations, prior to the electrophysiological experiments, we also demonstrated the presence of normal CaTs in multicellular papillary muscles. The shape of the recorded CaTs was close to those obtained from single isolated cardiomyocytes. The effects of $[Ca^{2+}]_o$ modulation on CaTs (systolic and diastolic levels) were also evaluated. Elevation of $[Ca^{2+}]_o$ to 4 mM resulted in 35 ± 11 % increase in the amplitude of the CaT. In contrast, the elevation in diastolic value was below the experimental variance. These results clearly demonstrate that the multicellular AP measurement technique is suitable for investigation of Ca^{2+} induced alterations in the AP.

Effect of apamin on APs recorded from canine, rat, and human multicellular cardiac preparations

The effect of 100 nM apamin on the kinetics of AP was tested using the conventional microelectrode technique in right ventricular papillary muscles and left atrial trabeculae excised from canine, rat, and undiseased human hearts. None of the preparations responded to apamin exposure with alterations in APD.

Since activation of the SK channels is tightly dependent on elevation of the cytosolic free Ca^{2+}, the effect of 100 nM apamin on the AP configuration was also evaluated in canine and rat right ventricular papillary muscles paced with varying frequencies. Representative AP pairs recorded from the same preparation before and after exposure to apamin, indicate that the APD was not modulated by apamin at any pacing frequency. Average canine APD_{90} values were 215±5.0 *versus* 219±4.0 ms at 0.3 Hz, 207±3.0 *versus* 209.5±3.5 ms at 1 Hz, and 188.0±3.0 *versus* 188.5±3.5 ms at 3 Hz, respectively, before and after apamin (n=5). The respective values in rat preparations were 83.8±7.1 *versus* 83.2±8.1 ms at 0.3 Hz, 73.0±3.5 *versus* 72.6±2.5 ms at 1 Hz, and 71.2±2.9 *versus* 72.6±3.4 ms at 3 Hz (n=5).

Representative CaTs were recorded from right ventricular tissues of dogs and rats, paced at 0.3, 1, and 3 Hz. The highest $[Ca^{2+}]_i$ values were observed at 3 Hz in canine myocytes, while the lowest at 0.3 Hz in the case of rat ventricular tissues, following the predictions of the positive and negative staircase phenomena, respectively, known to be characteristic of canine and rat ventricular myocardium. In spite of the elevated $[Ca^{2+}]_i$ values seen at these frequencies no effect of apamin on AP duration was observed.

In order to exclude the possibility that a strong repolarization reserve may fully compensate for any apamin-induced APD lengthening, prior to the application of apamin we also blocked the major repolarizing currents. These interventions are known to augment changes in APD induced by the blockade of other outward currents. In canine papillary muscles I_{Kr} was blocked by 300 nM dofetilide, in rat preparations 5 µM AVE0118 was used to inhibit the I_{to} plus I_{Kur}.

While a substantial attenuation of the repolarization reserve was obtained in the presence of these drugs, apamin exposure failed to alter APD even under these circumstances. In canine papillary muscles APD_{90} was increased by 300 nM dofetilide from 211±3.0 to 245±5.7 ms ($p<0.05$, n=5), and 247±6.0 ms was measured when 100 nM apamin was superfused in the presence of dofetilide. The respective values in the case of rat preparations were: 70.8±5.2 ms in control, 148.2±7.3 ms in the presence of 5 µM AVE0400 ($p<0.05$, n=5), and 148.4±7.0 ms following further exposure to 100 nM apamin.

The NCX current was measured in dog, rabbit and rat ventricular cardiac myocytes. In all species the selective NCX inhibitor SEA0400 (1 µM) markedly reduced NCX current. In spite of these results, 1 µM SEA0400 failed to influence the AP kinetics in all species, obtained from intact papillary muscles.

Effect of apamin on ion currents in single canine and rat ventricular cells

Under whole cell conditions, the free Ca^{2+} concentration in the pipette solution was set to 900 nM in order to induce maximal activation of SK channels. Test depolarizations, arising from the holding potential of -50 mV, were applied to membrane potentials ranging from -120 to +60 mV for 150 ms. The "steady-state" current-voltage relations, obtained before and after the exposure to 100 nM apamin, were fully identical in both canine and rat ventricular cells indicating that apamin failed to activate any ion current in these myocytes throughout the entire voltage range tested.

Under perforated patch clamp conditions the cytosol was prevented from dialysis. The cells were loaded with the fluorescent dye fluo 4AM to monitor the Ca^{2+} release simultaneously to membrane current measurements. The membrane was initially depolarized from a holding potential of -50 mV to +40 mV, then it was hyperpolarized to -100 mV (at a rate of 175 mV/s). Upon depolarization CaTs could be clearly observed indicating normal functional Ca^{2+} homeostasis in these cells. However, the steady-state membrane current failed again in either species to alter following the administration of 100 nM apamin.

The effect of increased $[Ca^{2+}]_i$ on I_{K1}

The effect of $[Ca^{2+}]_o$ on AP characteristics

APs from canine were determined under normal (2 mM) $[Ca^{2+}]_o$ or elevated (4 mM) $[Ca^{2+}]_o$ conditions. Under normal conditions the kinetics of APs was as expected (APD_{90}: 202.38±3.48 ms; APD_{50}: 154.25±3.47 ms; APD_{25}: 118±3.57 ms). Elevated $[Ca^{2+}]_i$ caused significant AP shortening without interfering the resting membrane potential (APD_{90}: 185.2±8.18 ms; APD_{50}: 121.8±1.93 ms; APD_{25}: 76.2±5.85 ms). Furthermore, in the presence of 4 mM $[Ca^{2+}]_o$ the velocity of the terminal repolarization was also increased.

The effect of 10 µM $BaCl_2$ on the AP

A selective I_{K1} blocker (10 µM $BaCl_2$) was used to evaluate the effects of $[Ca^{2+}]_o$ modulation on the current. At normal $[Ca^{2+}]_o$ and pacing at 1 Hz $BaCl_2$ lengthened both APD_{90} (202.38±3.48 ms *vs* 222.88±2.8 ms; Δ10.84±0.84%;$p<0.05$) and APD_{50} (154.25±3.47 ms *vs* 162.88±3.39 ms) however, APD_{25} was statistically unaffected (118±3.57 ms *vs* 121.75±3.39 ms). The resting membrane potential also remained unchanged. $[Ca^{2+}]_i$ rise following $[Ca^{2+}]_o$ elevation to 4 mM increased significantly the effect of 10 µM $BaCl_2$ on APD_{90} (185.2±8.18 ms *vs* 231.20±13.14 ms; Δ=19.6±1.9%;$p<0.05$) and APD_{50} (121.8±1.93 ms *vs* 141.8±3.99 ms) compared to normal settings, but did not affect resting membrane potential.

The effect of $[Ca^{2+}]_o$ elevation on I_{K1} inhibition was also analyzed in paired experiments: following the first application of 10 µM $BaCl_2$ under normal conditions $BaCl_2$ was completely washed out, $[Ca^{2+}]_o$ was raised up to 4 mM and $BaCl_2$ was reapplied. The effect of selective I_{K1} inhibition on APD_{90} lengthening was clearly augmented.

$[Ca^{2+}]_i$ dependent effect of I_{K1} on repolarization reserve

If I_{K1} was really increased by elevation of $[Ca^{2+}]_o$ (and consequently $[Ca^{2+}]_i$), contribution of I_{K1} to the repolarization reserve should also be increased under these conditions. This implicates that the lengthening effect of suppression of another K^+ current is expected to be smaller in high than in low $[Ca^{2+}]_o$. Indeed, as shown in, 0.3 µM dofetilide, known to fully block I_{Kr}, caused significantly smaller lengthening of APD_{90} when the preparation was exposed to dofetilide in the presence of 4 mM instead of 2 mM $[Ca^{2+}]_o$. The finding that dofetilide induced APD lengthening was attenuated at high $[Ca^{2+}]_o$ is crucially important, since it also indicates that the high $[Ca^{2+}]_o$-induced AP shortening cannot be related to faster activation of I_{Kr} due to the elevated plateau potential.

$[Ca^{2+}]_i$ dependent modulation of steady-state I_{K1}

The possible effect of $[Ca^{2+}]_i$ on I_{K1} was investigated by comparing the effect of low (~160 nM) and high (~900 nM) $[Ca^{2+}]_i$ directly on I_{K1} current by applying an appropriate mixture of $CaCl_2$ and Ca^{2+} chelator BAPTA. The Ca^{2+} levels in the pipette solution were verified by a Ca^{2+} sensitive electrode. The steady-state current was determined as the Ba^{2+} sensitive current at the end of 300 ms long rectangular voltage pulses ranging between -80 and -30 mV arising from a holding potential of -80 mV. The amplitude of I_{K1} was significantly augmented by high $[Ca^{2+}]_i$ between -70 and -40 mV ($p<0.05$).

Determination of the instantaneous I_{K1} current during an AP

In this set of experiments the instantaneous I_{K1} current was determined during an AP used as command potential. I_{K1} was again dissected from the total current by applying 10 μM $BaCl_2$. Applying high $[Ca^{2+}]_i$ in the pipette solution significantly increased the peak current, while the activation kinetics of the current remained unchanged. Furthermore, the I-V curve was slightly shifted toward positive direction when elevated $[Ca^{2+}]_i$ was applied in the pipette solution (from -65±1.1 to -57±1.3; $p<0.05$).

Effect of dynamic increase of $[Ca^{2+}]_i$ on steady state I_{K1}

Finally we tested the hypothesis that dynamic increase in $[Ca^{2+}]_i$, i.e. when CaTs were evoked during each cycle, may augment the I_{K1}. The currents dissected by 10 μM $BaCl_2$ were compared in the presence and absence of dynamic Ca^{2+} elevation. The voltage protocol started with a brief, 50 ms long prepulse from -80 mV to 0 mV in order to trigger CaT before current activation.

In the presence of Ca^{2+} buffering neither CaT nor current activation could be observed. In contrast, when dynamic Ca^{2+} elevation was allowed, I_{K1} increased markedly. When $[Ca^{2+}]_i$ was low, the I-V curve of I_{K1} was moderate, however, when EGTA was omitted from the pipette, the magnitude of the current was, again, considerably augmented between -70 to -30 mV ($p<0.05$).

DISCUSSION

Novel findings

In this thesis several aspects of the interactions between cardiac repolarization and intracellular Ca^{2+} handling are summarized. In the corresponding studies we attempted to collect novel information on the level of modulation of the cardiac AP by changes in $[Ca^{2+}]_i$, primarily focusing at the repolarization process. Two well known Ca^{2+}-dependent K^+ currents were investigated in details: 1) the small conductance Ca^{2+} activated K^+ current **(I_{SK})**, and 2). the inward rectifier K^+ current **(I_{K1}).** The results of the first study led us to the conclusion that the I_{SK} has no apparent role in the physiological repolarization process. Nonetheless, the potential contribution of this current to accelerated repolarization in certain pathological conditions cannot be ruled out. In the other study we conclude that the magnitude of I_{K1} is significantly enhanced, when $[Ca^{2+}]_o$ (and consequently $[Ca^{2+}]_i$) is largely elevated.

Are there functioning SK channels in mammalian myocardium?

Based on their high Ca^{2+}-sensitivity and relatively weak voltage-dependence, one could expect that SK channels provide an important functional

direct link between Ca^{2+} handling and the electrical events of the surface membrane. Thus, these channels might modify AP configuration by responding to beat-to-beat alterations of $[Ca^{2+}]_i$.

Previous results

In a previous study Xu et al. found that SK channel blockade by apamin (50 pM) significantly lengthened the APD in murine atrial and ventricular myocytes and also in human atrial cells. Implications of these observations may be very important, since the reported apamin-sensitive, marked lengthening of the APD suggests a crucial involvement of the SK channels in the physiological repolarization process. In addition, based on the observation that the APD is lengthened by its inhibition, modulation of I_{SK} may represent a novel antiarrhythmic mechanism, which could initiate novel pharmacological strategies. Furthermore, SK channels can be expected to substantially contribute to the repolarization reserve, especially during Ca^{2+} overload. Based on data from genetically altered mouse models, SK channels may have functional importance in development of atrial fibrillation and in the kinetics of the AV node APs.

Has any role SK current in cardiac repolarization?

Based on classical molecular biologic techniques, we also verified the expression and mostly plasmamembrane location of the SK2 channels, however, in contrast to findings by Xu et al, we failed to identify any apamin induced alteration either in ventricular or in atrial AP, as well as in the corresponding ion currents, in either species, studied. The major discrepancy between their and our results may either derive from interspecies differences, or from partially different experimental conditions. Perhaps the most important difference can be found in the AP measurements. The AP measurements by Xu et al were carried out in single isolated cardiomyocytes, while ours were performed in multicellular cardiac tissue using the conventional microelectrode technique. Recording APs from single isolated cells is a widely used/accepted technique, however, it may not be fully suitable for detection of small changes in APD, since the enzymatic dissociation process may result in compromised repolarization reserve. During isolation, a varying portion of K^+ channels are impaired, resulting in suppressed K^+ currents, quite similar to nonspecific K^+ channel inhibition. This may lead to AP instability, unexpected time dependent changes and attenuation of the effect of an ion channel blocker.

An alternative explanation for the found discrepancy may be that the physiologically achieved $[Ca^{2+}]_i$ level increase is not sufficient to activate large enough SK current, which could overcome the strong repolarizing capacity of the ventricular muscle. To clarify this possibility we attenuated the repolarizing

reserve by inhibiting major repolarizing currents (I_{Kr} in the dog, I_{Kur} plus I_{to} in the rat) prior to apamin administration. As expected, these interventions markedly lengthened the APD in both species, however, subsequently applied apamin still failed to induce any further APD prolongation, suggesting negligible role for apamin-sensitive SK-current in cardiac ventricular repolarization.

The negligible contribution of NCX current to the APD may rule out the possibility that the NCX current further complicate the evaluation of the SK-current.

A set of measurements were also performed in order to study the possible modulatory role of $[Ca^{2+}]_i$ alterations on the apamin effect. Our fluorometric measurements revealed that in canine cardiomyocytes the highest $[Ca^{2+}]_i$ levels could be reached at 3 Hz, while in rat ventricular myocytes at 0.3 Hz stimulation rate. No frequency-dependent differences in AP kinetics following apamin administration could be observed, suggesting that even the highest physiological $[Ca^{2+}]_i$ levels might still be too low to activate the SK channels.

In a set of further experiments we attempted to directly identify the apamin sensitive current in voltage clamped single canine and rat cardiomyocytes. However, application of 100 nM apamin failed to cause any shift of the control I-V curve in either species, again suggesting the lack of functional apamin-sensitive K^+ channels in ventricular myocardium. The lack of effect of apamin in our patch clamp experiments was unexpected, since Xu et al. reported rather large apamin-sensitive current (4 pA/pF) in the membrane potential range corresponding to the AP plateau.

The perforated patch clamp technique allows the preservation of the intracellular milieu of the cell, while CaTs and cell contraction are evoked. Therefore, this setting allows to investigate, whether the SK current only responds to dynamic $[Ca^{2+}]_i$ changes. However, 100 nM apamin failed again to cause any shift in the I-V curve.

What may be the message of our results?

In contrast to previous reports, our data seem to contradict the significance of the previous observations made in mice and in human, and the reason of this discrepancy is not clear. We must conclude that SK channels play only negligible - if any - role in cardiac repolarization under physiological conditions. However, the possibility that these channels may activate under a few special circumstances (such as Ca^{2+} overload, heart failure, or conditions of ischemia/reperfusion) cannot be ruled out. Our data may also have important implications regarding pharmacological speculations and future drug development. Further experiments

are required to investigate the putative role of the SK channels in diseased myocardium, before its pharmacological modulation could be utilized.

Ca^{2+} dependence of I_{K1}

I_{K1} is activated in the final phase of the repolarization process and sets and stabilizes the resting membrane potential. The current shows strong inward rectification, thus enables significantly larger inward than outward current. In outward direction, the current reduces close to zero at potentials positive than -30 mV, then quickly reopens when membrane potential approximates more negative values. This feature is due to Mg^{2+} and polyamines (spermine and spermidine) induced channel block upon depolarization. I_{K1} beyond being an important component of the repolarization reserve, also opposes any kind of arrhythmogenic depolarization occurring in rest, like the Ca^{2+} overload induced transient inward current. Hence, any impairment of this current may be arrhythmogenic, since positive resting membrane potential shifts facilitate extrasystoles. Furthermore, decreased I_{K1} lengthens APD and may increase the dispersion of repolarization, providing substrate for reentries. Although partial blockade of I_{K1} by 10 µM $BaCl_2$ substantially inhibits the current (about 70%) it only causes marginal APD lengthening. Therefore, under physiological conditions I_{K1} can be more considered as a component of the reserve than possessing major repolarizing role. Nevertheless, when repolarization reserve was attenuated, the effect of I_{K1} inhibition was found to be largely enhanced, indicating that in this circumstances I_{K1} can be a key player of the repolarization reserve.

I_{K1} mediated $[Ca^{2+}]_i$ effects on AP morphology

We investigated the putative Ca^{2+}-dependence of I_{K1} under multicellular, as well as single cell conditions. In the AP measurements we attempted to raise $[Ca^{2+}]_i$ as selectively as possible. To avoid activation of PKA and PKC, we did not use adrenergic agonists, thus the multiple effects of protein kinases may have averted. Instead, we increased $[Ca^{2+}]_o$ to 4 mM, thus enhancing the "driving force" for Ca^{2+} ions through the I_{CaL}. As expected, enhanced $[Ca^{2+}]_i$ markedly shortened APD_{90}. Furthermore, the maximal velocity of the terminal repolarization was also found to be faster. This effect may be a consequence of decreased inward and increased outward currents. Enhanced inactivation of I_{CaL} during high $[Ca^{2+}]_i$ conditions is a widely accepted mechanism underlying the AP shortening, however in the potential range of the terminal repolarization I_{CaL} is already inactivated.

Increased repolarizing currents (I_{Kr}, I_{Ks}, I_{K1}) were also suggested to contribute to enhanced terminal repolarization, especially when APD shortening is paralleled by elevated plateau potential. Therefore, we tested the putative role of the delayed rectifiers (I_{Kr} and I_{Ks}) in high $[Ca^{2+}]_i$ induced APD shortening. Interestingly, selective inhibition of I_{Kr} by dofetilide did not result in increased

APD lengthening when $[Ca^{2+}]_o$ was elevated. In contrast, the effect of dofetilide was significantly reduced. Following selective inhibition of I_{Ks} by HMR-1556 no effect could be observed. This results exclude a possible I_{Kr} enhancement by elevated $[Ca^{2+}]_i$, however, the reduced effect of dofetilide may be a result of the shortened APD_{90} caused by high $[Ca^{2+}]_i$. APD abbreviation is able to reduce the effect of I_{Kr} inhibition, however, it may only partially explain our findings, since under the same conditions the effect of I_{K1} inhibition was largely enhanced. An alternative explanation can be based on the concept of the repolarization reserve: i.e. the reduced effect of I_{Kr} inhibition could also be the consequence of increased outward currents (e.g.: I_{K1}), which is able to partially compensate for I_{Kr} inhibition.

In contrast to the negative results of I_{Kr} and I_{Ks} inhibition, the effect of I_{K1} inhibition was considerably increased by enhanced $[Ca^{2+}]_o$ in canine papillary muscles.

The effect of $[Ca^{2+}]_i$ on I_{K1} current

Since I_{K1} is permanently active during the late repolarization phase and the entire diastolic period, if I_{K1} was Ca^{2+}-dependent, both systolic and diastolic $[Ca^{2+}]_i$ would affect the magnitude of the current. In these experiments Ca^{2+} dependency of I_{K1} was compared between the high and low $[Ca^{2+}]_i$ groups and three sets of measurements were performed. In the first set steady-state currents were measured at the end of each voltage steps, in the second set the instantaneous currents were determined. Finally, in the third set the effect of dynamically increased $[Ca^{2+}]_i$ on the steady-state current was investigated. In all 3 sets of experiments I_{K1} was defined as *the* Ba^{2+} sensitive current.

Independent of the protocol used, following $[Ca^{2+}]_i$ rise the Ba^{2+}-sensitive current was substantially increased between -70 to -40 mV.

Has I_{K1} multiple regulation?

Several studies reported that beyond being regulated by $[Ca^{2+}]_i$, I_{K1} is also sensitive to alterations in the phosphorylation/dephosphorylation state of the channel proteins caused by a variety of protein kinases. Several studies reported that PKA, PKC and CaMKII may have distinct modulatory effects on I_{K1}. During adrenerg stimulation PKA and PKC may reduce the amplitude of I_{K1}, while CaMKII may augment the current. Indeed, it seems feasible that distinct modulatory pathways (PKA, PKC, CaMKII, $[Ca^{2+}]_i$) concomitantly target the corresponding channels and jointly fine-tune the amplitude of I_{K1} to improve the adaptation of the current to varying external conditions. Our results support that under high $[Ca^{2+}]_i$ conditions I_{K1} is enhanced by CaMKII. Sympathetic stimulation activates the PKA and PKC pathways both exerting crucial role in controlling the shape of the AP *via* modulating a few ionic currents like I_{CaL}, I_{Ks}, and I_{Cl}. Nonetheless, their inhibiting effect on I_{K1} can be seriously arrhythmogenic *via*

reducing the repolarization reserve and compromising the resting membrane potential. Therefore, simultaneous CaMKII activation and $[Ca^{2+}]_i$ increase may counteract and limit the PKA/PKC induced I_{K1} reduction, and thus prevent arrhythmia generation. Consequently, substantially different experimental conditions may cause the loss of the balance between these two counteracting pathways resulting in reduced I_{K1} amplitude. This explanation may help in understanding the contradicting results on Ca^{2+}-sensitivity of I_{K1}.

What may be the message of our results?

Overall, our data suggest that increased $[Ca^{2+}]_i$ may activate an autoregulative I_{K1} augmentation. Under conditions investigated, the enhancement in I_{K1} seems to be confined to the systolic period of the cardiac cycle. During diastole the resting membrane potential is practically not affected. Increased $[Ca^{2+}]_i$ is a principal component of the cardiac adaptation process, however, it may also lead to increased probability of arrhythmogenesis. Therefore, when $[Ca^{2+}]_i$ is elevated, a compensatory feed-back increase in I_{K1} should have antiarrhytmic role *via* enhancing the repolarization reserve. Considering disparate results in the literature, we could not rule out any direct enhancing effect of $[Ca^{2+}]_i$ on I_{K1}. Another possible mechanism for the $[Ca^{2+}]_i$-induced I_{K1} increase is *via* distinct activation of the CaMKII. Our results also suggest that elevated $[Ca^{2+}]_i$ may markedly influence the efficacy of class III antiarrhythmic drugs, counteracting their AP prolonging action.

CONCLUSIONS AND FURTHER PERSPECTIVES

Our results support the presence of a Ca^{2+} activated K^+ current in the membrane potential range of the terminal repolarization. However, we had to conclude that this current may not be the SK-current, rather the I_{K1}, which thus could improve the instantaneous adaptation of AP to an external challenge. Since I_{K1} cannot be related to one particular channel type, rather to a complex mixture of Ba^{2+} sensitive channels (primarily Kir2.x), active during terminal repolarization, we cannot exactly identify the primary Ca^{2+} sensitive ion channel(s).

We could not demonstrate an apamin sensitive K^+ current in cardiac ventricular muscle, and it is rather difficult to explain the obvious discrepancy between our results and data in the literature. However, considering the growing body of evidence on functional Ca^{2+} activated K^+ currents in the heart, and the relatively wide range of ion channels, which are inhibited by Ba^{2+}, we cannot rule out the possibility that the "classical" Ca^{2+} activated K^+ current and the Ca^{2+} sensitive I_{K1}, at least partially, overlap regarding the responsible ion channel(s). This hypothesis, however, requires further experimental work.

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