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The role of calcium-activated chloride current in cardiomyocytes

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Introduction

According to international statistics the prevalence of arrhythmias is 53 per 1000. In Hungary approximately 500 000 people suffer from arrhythmia. Arrhythmia is an important source of mortality in European countries and in Hungary too. 50 to 100 sudden unexpected cardiac deaths occur per 100 000 population every year. All disturbances of rhythm resulted from abnormalities in electrical activity of myocytes, because of the action potential (AP) morphology is altered or the activity of ion channels are abnormal. The abnormal activity causes abnormality in impulse initiation and/or abnormality in impulse propagation. Many of the antiarrhythmic drugs that are used to treat cardiac arrhythmias have their action on ion currents flowing transmembrane ion channels, and changes the process of repolarization, refractory period or the velocity of stimulus propagation. However, antiarrhythmic drugs have proarrhythmic (facilitation of new arrhythmias) effects.

Cardiac cation channels have been widely touted as the target of choice for the treatment of cardiac arrhythmia. However, drugs that target these channels have improved the survival of cardiac patients only moderately, highlighting a need for fresh approaches to antiarrhythmic therapy. Care must be taken applying antiarrhythmic drugs, because in several case proarrhythmic effects were reported. New targets, including Cl^- channels, have thus been identified as potentially important sites of action for future antiarrhythmic agents. The role and importance of cardiac Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) in shaping the AP is poorly understood. Previous studies have been investigated the role of Cl^- current in formation of delayed afterdepolarization and it has been demonstrated that Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) may be involved in development of arrhythmias. Therefore, understanding the role of Ca^{2+}-activated Cl^- current in shaping AP may create an opportunity to develop therapeutic agents against arrhythmias.

Aims

The exact role of Cl^- channels in cardiac electrical activity is unclear. $I_{Cl(Ca)}$ may contribute to the repolarization as I_{to2}, however the true profile of $I_{Cl(Ca)}$ during ventricular AP is poorly understood. Our aims were:

- to confirm the presence of $I_{Cl(Ca)}$ in canine left ventricular myocytes;
- to compare the effects of two I_{ClCa} inhibitors, 9-anthracene carboxylic acid (9-AC) and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS);
- to explore whether DIDS or 9-AC is preferable to study $I_{Cl(Ca)}$;
- to study the Ca^{2+}- and voltage dependence of $I_{Cl(Ca)}$;
- to characterize $I_{Cl(Ca)}$ profiles under actual action potential;
- to reveal the expression pattern of a $I_{Cl(Ca)}$ potential channel proteins, TMEM16A and Bestrophin-3 in human and canine myocytes;
- to visualize cellular appearance and co-localization of TMEM16A and Bestrophin-3 with each other as well as with pore forming subunit of L-type Ca^{2+} channels ($Ca_v1.2$).

Materials and methods

Electrophysiology

Experiments were performed using enzymatically isolated canine left ventricular myocytes. 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. Cell isolation was carried out by enzymatic dispersion using the segment perfusion technique. Action potentials (AP) were recorded using current clamp mode of patch clamp technique. Transmembrane ionic currents were recorded using the whole-cell configuration of the patch-clamp technique and action potential voltage-clamp (APVC) techniques. $I_{Cl(Ca)}$ was defined as an inhibitor-sensitive current ($I_{9\text{-}AC}$ or I_{DIDS}) obtained by pharmacological subtraction. Ion currents were normalized to cell capacitance.

During APVC experiments sequential dissection experiments were conducted. APs were recorded in current-clamp mode from the myocytes superfused with Tyrode solution and were used as a command in voltage-clamp mode. All experiments except for molecular biological techniques were applied at 37 °C.

Isolation of left ventricular myocytes from non-diseased human donor hearts

Human hearts were obtained from organ donors whose non-diseased hearts were explanted to obtain pulmonary and aortic valves for transplant surgery. The investigations conformed to the principles of the Declaration of Helsinki. Experimental protocols were approved by the Ethical Review Board of the Medical Center of the University of Szeged and by the Scientific and Research Ethical Committee of the Medical Scientific Board at the Hungarian Ministry of Health. Single, isolated left ventricular myocytes were used for immunocytochemistry and confocal imaging.

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

Single cell shortening was recorded by optical edge detection, while changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were assessed by FURA-2-AM.

Immunocytochemistry and confocal imaging

Immunolabeling for $Ca_v1.2$, TMEM16A and Bestrophin-3 was performed in isolated canine left ventricular myocytes and isolated human left ventricular myocytes. Cells were probed with anti-TMEM16A (rabbit, 1:200), anti-Bestrophin-3 (goat, 1:200), anti-$Ca_v1.2$ (rabbit, 1:200) anti-ryanodine receptor (mouse, 1:200), or with anti-cytochrome c (mouse, 1:200) primary antibodies. The secondary antibody labeling was the following: biotinylated anti-rabbit, anti-mouse or anti-goat antibody then Streptavidine Alexa488 incubation and with Cy3 anti-mouse or anti-rabbit labeling. To visualize the nuclei, cells were mounted with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI). The Pearson correlation coefficient was used to quantify the degree of co-localization between fluorophores.

Statistics

Results are expressed as mean ± SEM values. Statistical significance of differences was evaluated using one-way ANOVA followed by Student's *t*-test. Differences were considered significant when p was less than 0.05.

Results

Calcium-activated chloride current measured as either DIDS- or 9-AC-sensitive current is identical during conventional voltage-clamp recording

Conventional voltage-clamp technique was applied to investigate $I_{Cl(Ca)}$ in canine left ventricular cardiomyocytes. The current was measured with the application of the two well-known inhibitors: 0.2 mmol/l DIDS or 0.5 mmol/l 9-AC. Cells were clamped to -40 mV as the holding potential and the current was activated by 200 ms long voltage steps applied between -100 and +100 mV with 20 mV increments in every 5 sec. DIDS- or 9-AC-sensitive current was determined by deducting the current traces measured in the presence of the inhibitor from those recorded in the absence of them and normalized to cell capacitance. Both DIDS-sensitive and 9-AC-sensitive current traces showed rapid activation and reached their peak values within approximately 15 ms. Interestingly, not only the activation, but the decay of the current was also fairly rapid. The current decreased to near zero 100 ms after the onset of the depolarizing pulse. Currents activated at potentials higher than -40 mV and its current-voltage relationship had a bell-shaped curve with a peak value at +60 mV. The current-voltage relationships in case of the two tested drugs were practically identical.

Comparison of the effects of DIDS and 9-AC on the action potential measured with sharp microelectrode

Current-clamp studies were conducted in canine left ventricular cardiomyocytes where the actions on various parameters of the AP were determined. APs were recorded at 5 different steady-state cycle lengths in control condition. This was followed by the application of either 0.2 mmol/l DIDS or 0.5 mmol/l 9-AC. After the changes induced by the inhibitors reached a steady level (usually within 7-8 min) the AP measurements at the different cycle lengths were repeated. As the last step, the washout of the inhibitors was performed to test the reversibility of the

changes. The contours of the APs were modified by the two inhibitors in a similar fashion except the upstroke of the AP. AP duration was determined at the level of 90 % repolarization (APD_{90}) and increased by both inhibitors in a similarly, reverse rate-dependent manner. Phase-1 magnitude (determined as the difference between the membrane potential values of the peak and the deepest point of the notch) was reduced by both inhibitors but the cycle length dependence of the magnitude of this reduction was different. There was a greater reduction at long cycle length with 9-AC whereas the opposite was the case for DIDS, leading to a significantly greater reduction at 2 and 5 s long cycle lengths compared to that seen with 9-AC. Both the amplitude of the AP and the maximal rate of the depolarization (V_{max}) were reduced by only DIDS whereas 9-AC did not influence these parameters. The values of the resting membrane potential were not altered by either DIDS or 9-AC.

Dose-dependent effects of 9-AC on the action potential

Current-clamp studies were continued to determine whether the previously applied dose of 9-AC was sufficient to achieve maximal inhibition of $I_{Cl(Ca)}$. In these experiments the stimulation cycle length was 1 s and increasing concentrations of 9-AC were applied in a cumulative manner in the range of 0.05-1 mmol/l. It took 7-8 min to reach steady-state actions of the inhibitor, therefore at least 8 min of perfusion period was used for each dose. The previously seen reduction of phase-1, increases in plateau potential and APD_{90} values evoked by 9-AC developed gradually by increasing concentrations of the inhibitor. The smallest dose evoking significant action on both APD_{90} and phase-1 values was 0.3 mmol/l. The application of 1 mmol/l 9-AC did not increase the changes of AP parameters evoked by the previous dose of 0.5 mmol/l 9-AC any further.

Effects of 9-AC on various cardiac cation currents measured by conventional voltage-clamp

The previous current-clamp studies showed the similarities as well as the difference in the actions of DIDS and 9-AC on the AP of canine left ventricular myocytes. The reduction of AP amplitude and V_{max} in case of DIDS suggested the inhibition of sodium channels by that inhibitor. This action was not seen with the application of 9-AC even in the highest applied dose (1 mmol/l). To further test the possible non-selective actions of 9-AC the major cation currents were measured in control conditions and after the application of 10 min of 0.5 mmol/l 9-AC.

The amplitude of $I_{Ca,L}$ was unaltered by 9-AC. In the 6 studied cells $I_{Ca,L}$ density was -5.34±0.44 A/F in control condition and -5.27±0.44 A/F in the presence of 9-AC. I_{Kr} was assessed as tail current amplitudes recorded following repolarization to the holding potential. The amplitude of the I_{Kr} tail currents was unaffected by 9-AC. In the 4 studied myocytes 0.73±0.05 A/F was the density of I_{Kr} tail current in control whereas 0.72±0.05 A/F in the presence of 9-AC. I_{Ks} tail currents were also unchanged during 10 min of 9-AC perfusion in the 5 myocytes examined being 1.66±0.03 and 1.61±0.05 A/F in control and in 9-AC, respectively. I_{K1} current was determined at the end of the pulse and showed no change in the 6 studied cells being -18.00±1.54 and -18.36±2.16 A/F in control and in 9-AC, respectively.

Ca^{2+}-dependent behavior of $I_{Cl(Ca)}$ assessed by conventional voltage-clamp

To confirm $I_{Cl(Ca)}$ dependence on $[Ca^{2+}]_i$, the $I_{Cl(Ca)}$ was recorded in the presence of two chelators having different Ca^{2+} binding kinetics and affinity. Application of 10 mmol/l BAPTA in the pipette solution without the addition of Ca^{2+} resulted in a great reduction of $I_{Cl(Ca)}$ making the current barely detectable at any test potential (0.07±0.01 A/F at +60 mV). In contrast, application of 10 mmol/l EGTA in the pipette – although reduced $I_{Cl(Ca)}$ (from 2.44±0.28 A/F to 1.49±0.27 A/F at +60 mV), – but failed to eliminate the current. In addition, the voltage-dependence

of $I_{Cl(Ca)}$ in the presence and absence of EGTA was essentially similar. The Ca^{2+}-dependence of $I_{Cl(Ca)}$ was further studied by application of a pipette solution designed to mimic the estimated peak systolic value of $[Ca^{2+}]_i$ (1.1 μmol/l free Ca^{2+}). This was achieved by adding BAPTA and appropriate amounts of Ca^{2+} to the pipette solution. The amplitude of $I_{Cl(Ca)}$ was significantly larger when $[Ca^{2+}]_i$ was set to 1.1 μmol/l (0.31±0.06 A/F at +60 mV) comparing to values obtained with 10 mmol/l BAPTA, however, it was much smaller than that measured without buffering $[Ca^{2+}]_i$. Applying 1 μmol/l nisoldipine, a selective inhibitor of L-type Ca^{2+} channels, further supported the Ca^{2+}-dependent nature of $I_{Cl(Ca)}$, since both L-type Ca^{2+} current ($I_{Ca,L}$) and $I_{Cl(Ca)}$ were eliminated by nisoldipine. These data are in line with the view that Ca^{2+}-entry through L-type Ca^{2+} channels (in addition to the concomitant Ca^{2+}-release from the sarcoplasmic reticulum (SR)) may be essential for the physiological activation of $I_{Cl(Ca)}$.

Influence of $[Ca^{2+}]_i$ on $I_{Cl(Ca)}$ recorded under action potential voltage-clamp (APVC) conditions

The influence of $[Ca^{2+}]_i$ buffering (by EGTA or BAPTA) as well as the preserved Ca^{2+}-homeostasis were further studied on AP configuration and on the simultaneously recorded calcium transients (CaTs). $I_{Cl(Ca)}$ and $I_{Ca,L}$ were studied using their specific blockers (9-AC and nisoldipine, respectively) during the AP applying sequential dissection of ionic currents under conditions of APVC. CaTs were completely abolished when 10 mmol/l of a Ca^{2+} buffer (BAPTA or EGTA) was added to the pipette solution, while characteristic CaTs could be observed without these buffers. Under these conditions intracellular Ca^{2+}-handling must be physiological in the absence of intracellular dialysis. In line with these results, unloaded cell shortening was equally abolished by either BAPTA or EGTA. Using BAPTA-containing pipette solution resulted in a massive prolongation of APs accompanied by a pronounced positive shift in the plateau potential. Phase-1 amplitude was decreased by BAPTA in a greater extent than EGTA. In contrast to

BAPTA, EGTA failed to increase the duration of APs. Similarly, the profile of nisoldipine-sensitive current (I_{NISO}) was strongly different in the presence of the two Ca^{2+} buffers. BAPTA increased both the amplitude and integral of I_{NISO} significantly, while EGTA had no such effect on these parameters. 9-AC dissected an early narrow outward current having a peak value of 1.62±0.06 A/F which overlapped the phase-1 repolarization of the command AP (appearing 5.5±0.6 ms after the peak of the AP and 5.1±0.6 ms before the end of phase-1 repolarization). The outward peak always appeared later than the inward peak of I_{NISO} by 6.4±0.5 ms when studied with sequential dissection on the same cells using 9-AC followed by nisoldipine. This outward peak of $I_{9\text{-}AC}$ was followed by a smaller late inward current reaching its maximum (−0.16±0.02 A/F) towards the end of the AP. BAPTA reduced significantly both the outward as well as the inward components of $I_{9\text{-}AC}$ - the outward current peak decreased nearly to zero. The effect of EGTA on the early outward current peak was smaller (1.17±0.09 A/F, $p<0.05$). These changes in current amplitudes were also reflected by similar alterations of current integrals. In summary, CaTs were completely eliminated by both Ca^{2+} buffers, while BAPTA but not EGTA abolished $I_{Cl(Ca)}$ - probably due to a more effective buffering of $[Ca^{2+}]_{cleft}$.

Effect of changes in $[Cl^-]_i$ on the profile of $I_{Cl(Ca)}$ measured with APVC

In the APVC experiments described above, intracellular Cl^- concentration ($[Cl^-]_i$) was set to 47 mmol/l, yielding a Cl^- reversal potential of −32 mV. In this case, without applying any Ca^{2+} buffer, $I_{9\text{-}AC}$ displayed initially an early outward component which was followed by a late inward component having peak densities of 1.62±0.08 A/F and −0.16±0.03 A/F, respectively, in the 15 cells studied. To test and reinforce the charge carrier, the concentration of Cl^- in the pipette solution was lowered to 27 mmol/l (by replacing Cl^- with equimolar aspartate) setting the new Cl^- reversal potential to −47 mV. This reduction of $[Cl^-]_i$ resulted in an enhancement of the amplitude and integral of the early outward component, while

the late inward component was practically eliminated. These data are considered as further supporting evidence that $I_{Cl(Ca)}$ was indeed mediated by Cl^-.

Study of $I_{Cl(Ca)}$ on two different stimulation frequency

$I_{9\text{-}AC}$ profiles were tested at two different steady-state pacing rates under APVC conditions. APD_{90} was increased by 9-AC in a reverse-rate dependent manner. According to the expectations, both systolic and diastolic $[Ca^{2+}]_i$ were higher when the preparations were stimulated at 2 Hz than at 1 Hz. In line with the larger CaT, the density of $I_{9\text{-}AC}$ as well as the total charge carried by the outward component of the current significantly increased when stimulatory frequency was increased from 1 Hz to 2 Hz.

$I_{Cl(Ca)}$ is increased by enhancement of $I_{Ca,L}$

As demonstrated earlier $I_{Ca,L}$ seems to play a critical role in the activation of $I_{Cl(Ca)}$. In the next experiments $I_{Ca,L}$ was enhanced by two different mechanisms. First a protein kinase A-dependent phosphorylation of the calcium channel protein was induced by the β-adrenergic agonist isoproterenol (ISO) applied in a concentration of 10 nmol/l. In another set of experiments direct pharmacological stimulation of $I_{Ca,L}$ was achieved by 20 nmol/l of Bay K8644. As it was confirmed with the recording of CaTs, both interventions resulted in an increased systolic $[Ca^{2+}]_i$, although ISO was more potent in this regard. ISO acted similarly to Bay K8644: the plateau potential was increased and phase-1 amplitude was reduced by both interventions. In contrast, opposite effects were observed on the duration of the AP, since AP was shortened by ISO while lengthened by Bay K8644. This can be attributed to the enhancement of I_{Ks} and I_{Kr} by ISO. I_{Ks} and I_{Kr} are not activated by Bay K8644 but $I_{Ca,L}$ is expected to be increased by both 10 nmol/l ISO and 20 nmol/l Bay K8644. $I_{Cl(Ca)}$ was dissected by 0.5 mmol/l 9-AC using the AP recorded in Tyrode solution as the command signal. Both inward and outward peaks of $I_{9\text{-}AC}$ were significantly larger after pretreatment with either 10 nmol/l ISO

or 20 nmol/l Bay K8644 as compared to untreated controls. In addition, its profile, its amplitude and the total charge carried by $I_{9\text{-}AC}$ were similar after these pharmacological interventions, irrespective of the way of $I_{Ca,L}$ enhancement. These results support the view that Ca^{2+}-entry through the L-type calcium channels is critically important in the activation of $I_{Cl(Ca)}$.

Blockade of SR Ca^{2+}-release by ryanodine fails to abolish $I_{Cl(Ca)}$

Contribution of Ca^{2+}-induced Ca^{2+} release (CICR) from the SR to the activation of $I_{Cl(Ca)}$ was also examined. Superfusion of the cells with 10 μmol/l ryanodine for 15 min greatly reduced both CaT and cell shortening. Ryanodine exposure also resulted in the elevation of the early plateau phase of AP. However, 0.5 mmol/l 9-AC applied in the presence of ryanodine caused a further reduction of phase-1 amplitude. Dissection of the corresponding ionic current by 9-AC under APVC conditions using the AP recorded in Tyrode solution as the command signal demonstrated a clear-cut $I_{Cl(Ca)}$ in the presence of ryanodine, having its usual profile, although its amplitude was significantly smaller than that measured under control conditions. Taken together, these data give strong support to our hypothesis stating that CICR is not essential for, but contributes to the activation of $I_{Cl(Ca)}$ which is rather influenced by transsarcolemmal Ca^{2+} entry.

Co-expression of TMEM16A, Bestrophin-3 and Cav1.2 in the t-tubules of ventricular cells

The expression pattern of potential channel protein candidates of $I_{Cl(Ca)}$ has been studied using immunocytochemistry and confocal microscopy. $Ca_v1.2$, the pore forming subunit of $I_{Ca,L}$, was also assessed. The specificity of the applied antibodies was tested by omitting the primary antibodies during the incubation procedure. According to expectations, $Ca_v1.2$ protein expression was most abundant in the t-tubules. Importantly, strong co-localization was found between $Ca_v1.2$ and Bestrophin-3 indicated by the 0.74±0.03 value of the Pearson

correlation coefficient (PCC) obtained in 8 cells. TMEM16A showed a similar expression pattern and strong co-localization with Bestrophin-3 with a PCC of 0.74±0.06 (n=8). In case of isolated human cells even stronger co-localization was detected than in case of canine cells. PCC for $Ca_v1.2$ and Bestrophin-3 yielded a 0.84±0.02 value obtained in 18 cells. TMEM16A showed a similar expression pattern and strong co-localization with Bestrophin-3 with a PCC of 0.85±0.02 (n=5).

TMEM16A, Bestrophin-3, RyR and cytochrome c expression patterns in canine cardiomyocytes

To exclude the possibility of an accidental co-expression and/or co-localization, the expression of channel protein candidates of $I_{Cl(Ca)}$ was assessed together with other proteins. First the expression of cytochrome c was tested with TMEM16A. In spite of the recognizable striated pattern of cytochrome c expression, the co-localization of cytochrome c and TMEM16A is unlikely as the merged image did not show strong overlap (PCC was 0.25±0.03 in 7 cells). Although the co-localization of Bestrophin-3 with RyR was slightly stronger (PCC = 0.36±0.05) in 3 cells, it was much weaker than with Cav1.2. Similarly, the co-localization of TMEM16A with RyR was not pronounced (PCC of 0.31±0.01) in 3 cells.

Discussion

The major finding of this study is that 9-AC is a better tool to study the physiological role of $I_{Cl(Ca)}$ in AP measurements compared to DIDS due to the lack of sodium channel inhibition in case of the former. 9-AC has also no effect on the other main ion currents ($I_{Ca,L}$, I_{Kr}, I_{Ks}, and I_{K1}) in ventricular myocytes. $I_{Cl(Ca)}$ was demonstrated to be active during the phase-1 and terminal repolarization. Our data gave further supporting evidence that $I_{9\text{-}AC}$ was Ca^{2+} activated and indeed mediated by Cl^-. Furthermore based on our results we can conclude that Ca^{2+}-entry through L-type Ca^{2+} channels is sufficient for the activation of $I_{Cl(Ca)}$ and does not require Ca^{2+}-release from the SR through RyR. Our results demonstrate the expression of Bestrophin-3 and TMEM16A in both canine and human ventricular myocytes for the first time and the strong co-localization of Bestrophin-3 with TMEM16A and $Ca_v1.2$ protein.

Comparing the effect of 9-AC and DIDS

Similar current amplitudes were recorded in the whole examined voltage range by both drugs. This indicates that $I_{Cl(Ca)}$ have the same magnitude measured either as 0.2 mmol/l DIDS- or 0.5 mmol/l 9-AC-sensitive current suggesting that the blocking potency of the two drugs is probably the same.

9-AC- and DIDS-sensitive current had a characteristic bell-shaped current voltage relationship in agreement with previous studies.

Interestingly, not only the activation, but the decay of $I_{Cl(Ca)}$ was also fairly rapid, as it decreased to near zero within 30 ms after the onset of the depolarizing pulse just as in previous studies. This phenomenon is quite opposite compared to that observed with expressed TMEM16A channels, where a maintained component also can be seen. This can be a result of the physiological $[Ca^{2+}]_i$ changes and the remarkable compartmentalization, which factors are much more pronounced in native cardiac myocytes.

Comparing the actions of the two studied inhibitors on various parameters of the AP the most important difference was the presence or absence of V_{max} reduction with DIDS or 9-AC, respectively. DIDS was not mentioned to reduce the V_{max} parameter in studies performed in rabbit or in canine myocytes on the contrary to the result of the present study. A potential explanation can be the different species or experimental conditions as in the rabbit study whole-cell patch clamp was done instead of the more physiological conventional sharp microelectrode recording of the present study.

As was demonstrated, the action of the two studied inhibitors on various AP parameters were largely identical (except those on V_{max} and AP amplitude), although slight differences in phase-1 and plateau potential changes induced by the two drugs were seen. A greater reduction of phase-1 was seen at long cycle lengths (2 and 5 sec) with 9-AC. The magnitude of phase-1 depends on many ion currents including I_{Na}, I_{to1}, $I_{Cl(Ca)}$ and $I_{Ca,L}$ and the precise timing of their activation, inactivation and reactivation. DIDS was shown earlier to reduce at least 3 of these (I_{Na}, $I_{Cl(Ca)}$ and $I_{Ca,L}$) whereas there is no evidence that 9-AC reduces any other current apart from $I_{Cl(Ca)}$. Moreover, the rate-dependence of these currents can complicate the picture as phase-1 magnitude itself shows reverse rate-dependence having larger values at longer cycle lengths mainly due to two factors working simultaneously. First, the slow recovery from inactivation of I_{to1}, which decreases the amplitude of I_{to1} at increasing heart rates, and secondly, $I_{Ca,L}$ increases with the reduction of cycle length in a Ca^{2+}-calmodulin protein kinase (CaMKII)-dependent regulatory pathway (also called as Ca^{2+} current facilitation), resulting in an earlier elevation of the AP plateau. Thus, the direct rate-dependent effect of 9-AC on phase-1 magnitude could be the result of clear-cut inhibition of $I_{Cl(Ca)}$, which current is increasing at higher stimulatory frequencies as it probably follows the frequency-dependent properties of the Ca^{2+} entry and CaTs. While the reverse-rate dependent effect of DIDS on phase-1 could be attributed to an almost constant I_{Na} blockade, reflecting the general feature of frequency-dependence of phase-1

magnitude. Therefore, the difference in rate-dependent inhibition of phase-1 by the two studied blockers can simply be due to weaker selectivity of DIDS. The reverse-rate dependency of AP lengthening after the application of either DIDS or 9-AC shows the intrinsic property of APD modulation rather than the frequency-dependence of the blocked ion current.

The effect of DIDS on plateau potential was small but statistically significant and did not depend on stimulation rate. On the contrary, 9-AC increased plateau potential in a direct rate-dependent manner resulting in the smaller effect of DIDS at short cycle lengths (0.3 and 0.5 sec). The weaker selectivity of DIDS can provide again a possible explanation as the reduction of $I_{Ca,L}$ likely causes the reduction of plateau potential (or more precisely the smaller increase of that) compared to 9-AC which was actually the case at all studied cycle lengths.

Concentration-dependent effect of 9-AC on ventricular AP was also studied. Concentrations of 9-AC were ranging from 0.1 to 1 mmol/l to achieve chloride channel inhibition in the literature. The reduction in phase-1 magnitude just as the lengthening of AP was saturated at 0.5 mmol/l of 9-AC as 1 mmol/l 9-AC did not increase these changes much further. It must be noted, however, that both parameters (phase-1 magnitude and APD_{90}) depend on not only one ionic current, but the fine-tuned interplay between several ionic currents, including I_{to1}, I_{Na}, and $I_{Ca,L}$ in case of phase-1 magnitude and even more in APD_{90}. From our results one can draw the conclusion that the application of 9-AC in 0.5 mmol/l dose is suitable to examine $I_{Cl(Ca)}$ as that dose completely inhibits the chloride current and its actions on the morphology of the AP are saturated.

In conclusion, there was no difference in the actions of DIDS and 9-AC on $I_{Cl(Ca)}$ examined with standard, rectangular voltage pulses. DIDS however significantly blocks fast sodium channels therefore it is not suitable in AP measurements, when the investigation focuses on the physiological role of $I_{Cl(Ca)}$ in the formation of APs. The difference between the action of the blockers on AP is becoming even more pronounced at increasing heart rates, resulting in the fact that DIDS but not

9-AC fails to demonstrate the frequency-dependent properties of $I_{Cl(Ca)}$. One must emphasize that DIDS is equally useful to study $I_{Cl(Ca)}$ during voltage-clamp when I_{Na} inhibition is not an issue due to the constant inactivated state of the channels.

Other major ventricular ion currents ($I_{Ca,L}$, I_{Kr}, I_{Ks}, and I_{K1}) were not affected by 9-AC in a dose (0.5 mmol/l) close to that completely blocks chloride channels.

Properties of $I_{Cl(Ca)}$

In the present study we aimed to characterize $I_{Cl(Ca)}$ in isolated canine ventricular myocytes under physiological conditions. This included the application of APVC allowing to record the current during the physiological AP waveform and using pipette solution without buffering of $[Ca^{2+}]_i$ allowing to develop CaTs with normal kinetics. This is critically important in the case of a Ca^{2+}-sensitive current, like $I_{Cl(Ca)}$. Although several studies described $I_{Cl(Ca)}$ in various cardiac preparations, the properties of $I_{Cl(Ca)}$ under true APVC conditions (i.e. when the current is studied during an AP recorded from the same myocyte) have not been analyzed.

The profile of $I_{Cl(Ca)}$, when studied as a $I_{9\text{-}AC}$ using APVC, was composed of a fast, early outward and a late inward component. The outward component showed a rapid activation as well as a remarkable rapid decay in spite of the continuously elevated $[Ca^{2+}]_i$ and the existing large driving force for Cl^- influx. One possible explanation for this rapid decay of $I_{Cl(Ca)}$ might be the time dependent inactivation of the channels mediating $I_{Cl(Ca)}$. It had been shown, however, that in the case of a constant $[Ca^{2+}]_i$ $I_{Cl(Ca)}$ showed a time-independent behavior upon prolonged depolarization making this option questionable. The same study also suggested the existence of a low-affinity Ca^{2+} binding site evoking inactivation of $I_{Cl(Ca)}$. The possible mechanism for Ca^{2+}-dependent inactivation (CDI) was studied in TMEM16A channels expressed in HEK cells, where phosphorylation by a Ca^{2+}-calmodulin dependent protein kinase II was suggested. This however is unlikely to mediate CDI during the AP. A more likely explanation for the rapid decay of $I_{Cl(Ca)}$

may be the rapid and massive reduction of $[Ca^{2+}]_{cleft}$, a change much faster and greater than observed with $[Ca^{2+}]_i$. This is in line with our results showing co-localized arrangement of the Ca^{2+} and Cl^- channels and allowing a direct guidance of $I_{Cl(Ca)}$ by $[Ca^{2+}]_{cleft}$, which is strictly controlled by the neighboring Ca^{2+} channels. The late inward component in the profile of $I_{Cl(Ca)}$ appearing towards the end of the AP also needs discussion. According to our hypothesis the current is activated due to the combination of membrane potential and an appropriate value of $[Ca^{2+}]_{cleft}$ which it is still able to activate $I_{Cl(Ca)}$ but not too high to cause calmodulin dependent inactivation. During terminal repolarization the membrane potential reduces below the equilibrium potential of Cl^- (−32 mV in our experiments except when low $[Cl^-]_i$ was used) therefore an inward current is generated through open channels by the efflux of Cl^-.

The source of the increase of $[Ca^{2+}]_{cleft}$ during diastole can be the spontaneous Ca^{2+}-release through ryanodine receptors in a Ca^{2+}-overloaded cell. The increase of $[Ca^{2+}]_{cleft}$ can result in formation of arrhythmogenic delayed afterdepolarizations (DAD) by forward mode operation of Na^+/Ca^{2+} exchanger (NCX) and $I_{Cl(Ca)}$ activation. Activation of $I_{Cl(Ca)}$ in canine ventricular cells as well as in ovine Purkinje cells and ventricular myocytes was responsible for DAD formation highlighting the pathophysiological significance of $I_{Cl(Ca)}$.

However, in a cell without Ca^{2+}-overload increase of $[Ca^{2+}]_{cleft}$ to a level where it can activate $I_{Cl(Ca)}$ at the end of the AP is difficult to explain. One option is the amplification of the window current of Ca^{2+} channels towards the end of the AP due to the growing driving force for Ca^{2+}-influx. $I_{Ca,L}$ mediated Ca^{2+}-influx due to the reactivation of L-type Ca^{2+} channels during terminal repolarization was observed in guinea-pig ventricular myocytes. In some canine ventricular cells a similar late inward peak of I_{NISO} measured in normal Ca^{2+} homeostasis was also present.

Another option is the release of Ca^{2+} from myofilaments at the end of the AP which is extruded via NCX and might also lead to the activation of $I_{Cl(Ca)}$.

Experimental evidence exists showing this late activation of forward mode NCX at the end of the AP in guinea-pig ventricular cells and also in canine cells. Indeed, we have observed similar timing of this inward I_{NCX} component and the final inward $I_{Cl(Ca)}$ recorded in the same cell.

Moreover, late Na^+ current is largest towards the late plateau of the AP measured in APVC during intact Ca^{2+} homeostasis. This Na^+-influx slows down the previously mentioned Ca^{2+}-extrusion via forward mode NCX therefore delays the reduction of $[Ca^{2+}]_{cleft}$ which might also contribute to the activation of $I_{Cl(Ca)}$.

Finally, the late inward component of $I_{Cl(Ca)}$ could be due to the reopening of Cl^- channels at the end of the AP. During the majority of the plateau phase of the AP TMEM16A can be inactive because high $[Ca^{2+}]$ inactivates TMEM16A via calmodulin. Upon the reduction of $[Ca^{2+}]_{cleft}$ Ca^{2+} can gradually dissociate from the low affinity binding site of calmodulin leading to the suspension of CDI of TMEM16A channels. In this scenario $[Ca^{2+}]_{cleft}$ can be large enough to activate $I_{Cl(Ca)}$ but lower than that which causes calmodulin mediated CDI.

Ca^{2+}-entry via $I_{Ca,L}$ is essential for the activation of $I_{Cl(Ca)}$, while Ca^{2+}-release from SR only augments $I_{Cl(Ca)}$

Previous studies emphasized the principal role of SR Ca^{2+}-release in the activation of $I_{Cl(Ca)}$ in rabbit atrial myocytes and Purkinje fibers. In contrast, we always observed large amplitude of $I_{Cl(Ca)}$ in canine myocytes in the continuous perfusion of 10 μmol/l ryanodine started 15 min prior to recording. One might argue that 15 min pretreatment is not enough to achieve the blockade of Ca^{2+}-release from SR but this is unlikely since both CaTs and cell shortening were largely abolished within 8-10 min of ryanodine perfusion. Further possible reason for the observed differences in ryanodine-sensitivity of $I_{Cl(Ca)}$ might be the different animal model used or other differences in experimental conditions. We applied canine ventricular cells in the present study in contrast to others using atrial myocytes and Purkinje

fibers of the rabbit. It is important to note that 9-AC was capable of decreasing phase-1 amplitude even in the presence of ryanodine. Furthermore, the profile of $I_{Cl(Ca)}$ recorded under APVC conditions in the presence of ryanodine was very similar to that seen without ryanodine further confirming that in our experimental setting Ca^{2+}-release from the SR is not an absolute requirement for the activation of $I_{Cl(Ca)}$. In contrast to the limited efficacy of ryanodine to eliminate $I_{Cl(Ca)}$ in our measurements, blockade of Ca^{2+}-entry through L-type Ca^{2+} channels by nisoldipine fully abolished $I_{Cl(Ca)}$.

Our other experiments support the importance of Ca^{2+}-entry through L-type Ca^{2+} channels further. The amplitude of $I_{Cl(Ca)}$ strongly increased when $I_{Ca,L}$ was enhanced either by ISO or Bay K8644. Both drugs increases $I_{Ca,L}$ but as Bay K8644 does not phosphorylate channel proteins (unlike what is the case in the presence of ISO). However, both treatments increased $I_{Ca,L}$ and $I_{Cl(Ca)}$ amplitudes to the same extent, while the concomitant elevations of CaT amplitudes were not proportional.

In line with this view, increasing the stimulation rate from 1 to 2 Hz also increased $I_{Cl(Ca)}$ probably due to the increased transsarcolemmal Ca^{2+} entry.

Co-localization studies have revealed that L-type Ca^{2+} channels and RyRs are in close proximity to one another within the dyadic cleft. During excitation-contraction coupling $[Ca^{2+}]_{cleft}$ is believed to be much higher and has faster kinetics than in the bulk cytoplasm. However, measuring $[Ca^{2+}]_{cleft}$ directly is just becoming available. According to our hypothesis, $I_{Cl(Ca)}$ is controlled locally, by $[Ca^{2+}]_{cleft}$, rather than bulk $[Ca^{2+}]_i$. This approach is supported by the experiments aiming to set $[Ca^{2+}]_i$ to 1.1 μmol/l (which corresponds to peak value of systolic $[Ca^{2+}]_i$) by dialyzing cells with pipette solution containing 1.1 μmol/l of free Ca^{2+}. In this case, $I_{9\text{-}AC}$ amplitude was much smaller, only 13 % of the value obtained without buffering $[Ca^{2+}]_i$. This is in a good agreement with the Ca^{2+}-sensitivity of $I_{Cl(Ca)}$ determined in murine airway smooth muscle, where the EC_{50} was estimated to be 3.3 μmol/l but only 20-50 μmol/l $[Ca^{2+}]_i$ resulted in full activation of the

current. Therefore, the significantly larger $I_{Cl(Ca)}$ measured under physiological conditions, i.e. without buffering $[Ca^{2+}]_i$, is probably due to the higher $[Ca^{2+}]_{cleft}$.

The different actions of EGTA and BAPTA on $I_{Cl(Ca)}$ and $I_{Ca,L}$ can also be explained by the ability of Ca^{2+} chelators to differentially affect $[Ca^{2+}]_i$ and $[Ca^{2+}]_{cleft}$. These agents suppress $I_{Cl(Ca)}$ and CDI of $I_{Ca,L}$ with different efficacy. Although both buffers eliminated CaTs, only BAPTA was effective to suppress CDI of $I_{Ca,L}$ resulting in significantly higher I_{NISO} amplitude and total charge values in addition to the much longer AP. BAPTA fully eliminated $I_{Cl(Ca)}$ during the AP, while EGTA only decreased its amplitude and the total charge carried by the current. These results are in a good agreement with the conventional voltage-clamp observations. The reason for the different buffering efficacy is that BAPTA has a 100 times faster binding rate to Ca^{2+} than EGTA. As mentioned previously, blockade of Ca^{2+}-release from the SR failed to fully abolish $I_{Cl(Ca)}$, only some reduction of the current amplitude was observed. It is rather difficult, however, to judge the relative contribution of Ca^{2+}-entry through L-type Ca^{2+} channels and Ca^{2+}-release from SR to the activation of $I_{Cl(Ca)}$. One reason is that the CDI of $I_{Ca,L}$ is known to be reduced when Ca^{2+}-release from SR is blocked (e.g. in the presence of ryanodine). Therefore the relative contribution of L-type Ca^{2+} channels to $I_{Cl(Ca)}$ activation is probably overestimated in the presence of ryanodine. Determining the precise contribution of these two mechanisms is further complicated by the presence of several other Ca^{2+}-dependent processes involved in the ventricular electrical activity, including the NCX, Ca^{2+}-dependent K^+ channels, and various Ca^{2+} pumps, likely to be altered by changes in $[Ca^{2+}]_i$ and even more by alterations in $[Ca^{2+}]_{cleft}$.

Co-localization of $Ca_v1.2$ with TMEM16A and Bestrophin-3 proteins, possible contributors of $I_{Cl(Ca)}$

In this study we tested the co-localization of Bestrophin-3 and/or TMEM16A, the two likely candidates responsible for $I_{Cl(Ca)}$, with $Ca_v1.2$ protein. The expression of Bestrophin-1 and Bestrophin-3 was demonstrated in murine heart. Moreover, when Bestrophin-1 or Bestrophin-3 were extracted and transfected to HEK cells, the resultant current recapitulated the characteristics of $I_{Cl(Ca)}$, including Ca^{2+}-dependence, anion selectivity and sensitivity to DIDS or niflumic acid. The same group reported the expression of Bestrophin-3 in human heart (although only at mRNA level in the latter) similarly to Bestrophin-1. The cellular expression of Bestrophin-3 in murine ventricular cells showed a punctuated pattern which was strong in the sarcolemma. In our images obtained in canine and human ventricular myocytes the cellular expression of Bestrophin-3 showed striated pattern rather than being punctuated.

Cardiac expression of TMEM16A has been described in murine ventricle, but this is the first report demonstrating it in canine and human ventricular preparations. In both species a clear-cut striated pattern was observed. Importantly, the expression of Bestrophin-3 showed strong co-localization with $Ca_v1.2$ channel protein and TMEM16A making it very likely that $Ca_v1.2$ and TMEM16A also co-localize with one another. In line with the results of the present study Ca^{2+}-activated Cl^- channels localize close to RyR and are controlled by local Ca^{2+} signals in airway smooth muscle. Bestrophins can associate with TMEM16A and contribute to the functional channel in vascular smooth muscle cells. This possibility, however, is very difficult to test since TMEM16A modulates Bestrophin expression. Down-regulation of TMEM16A reduced the expression of Bestrophins, however, a reduced expression of L-type Ca^{2+} channels has also been mentioned. On the other hand, Bestrophins were shown to form preferentially homomer channels, similarly to TMEM16A, which may question the ability of the two proteins to form a single functional ion channel.

The striated pattern of TMEM16A, Bestrophin-3, and $Ca_v1.2$ expression is most likely due to the strong expression of these proteins in the membranes of the t-tubules. Similar images were published in murine and human ventricular cardiomyocytes with $Ca_v1.2$ expression in the t-tubules. The accidental co-localization of the channel proteins due to technical factors of imaging is unlikely since the co-localization of TMEM16A with cytochrome c was weak. Similarly, less pronounced co-localization was seen between Bestrophin-3 and RyR than between Bestrophin-3 and TMEM16A/$Ca_v1.2$. The expression pattern of RyR was very similar to that seen in rat ventricular myocytes.

Summary

Understanding the role of ionic currents in shaping the cardiac action potential (AP) has great importance as channel malfunctions can lead to sudden cardiac death by inducing arrhythmias. Despite the possible contribution of ($I_{Cl(Ca)}$) may mediated by TMEM16A and/or Bestrophin-3 to cardiac arrhythmias, the true profile of $I_{Cl(Ca)}$ during an actual ventricular AP, is poorly understood.

In this study we aimed to explore which blocker, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) or 9-anthracene carboxylic acid (9-AC) is preferable to visualize the profile of $I_{Cl(Ca)}$. We aimed to study $I_{Cl(Ca)}$ at normal Ca^{2+} cycling and by AP voltage-clamp as well as in conditions designed to change $[Ca^{2+}]_i$ in canine ventricular myocytes. The expression of TMEM16A and/or Bestrophin-3 in canine left ventricular myocytes and human ventricular myocardium by immunolabelling was examined. The possible spatial distribution of these proteins and their co-localization with $Ca_v1.2$ was also studied.

Both inhibitors, DIDS (0.2 mmol/l) and 9-AC (0.5 mmol/l) increased AP duration. DIDS- and 9-AC-sensitive currents were identical in voltage-clamp conditions. 9-AC did not influence either the AP amplitude or the maximal rate of depolarizations (V_{max}), while DIDS caused marked reduction of V_{max}. The profile of $I_{Cl(Ca),}$ identified as a 9-AC-sensitive current under AP voltage-clamp conditions, contained an early fast outward and a late inward component, overlapping early and terminal repolarizations, respectively. Both components were moderately reduced by ryanodine, while fully abolished by BAPTA, but not EGTA. Setting $[Ca^{2+}]_i$ to the systolic level measured in the bulk cytoplasm (1.1 μmol/l) decreased $I_{Cl(Ca)}$. Increase in $[Ca^{2+}]_i$ elicited by application of BayK 8644, isoproterenol, and faster stimulation rates increased the amplitude of $I_{Cl(Ca)}$. We concluded that Ca^{2+}-entry through L-type Ca^{2+} channels is essential for activation of $I_{Cl(Ca)}$. TMEM16A and Bestrophin-3 showed strong co-localization with one another and also with $Ca_v1.2$ channels, when assessed using immunolabelling and confocal microscopy in both canine myocytes and human ventricular myocardium.

These results suggest that DIDS and 9-AC are equally useful to study $I_{Cl(Ca)}$ during voltage-clamp but 9-AC is superior in AP due to its lack of sodium channel inhibition. Activation of $I_{Cl(Ca)}$ in canine ventricular cells requires Ca^{2+}-entry through neighboring L-type Ca^{2+} channels and is only augmented by SR Ca^{2+}-release. Substantial activation of $I_{Cl(Ca)}$ requires high Ca^{2+} in the dyadic clefts which can be effectively buffered by BAPTA, but not EGTA.

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List of publications related to the dissertation

1. Horváth, B.*, **Váczi, K.***, Hegyi, B., Gönczi, M., Dienes, B., Kistamás, K., Bányász, T., Magyar, J., Baczkó, I., Varró, A., Seprényi, G., Csernoch, L., Nánási, P. P., Szentandrássy, N.: Sarcolemmal Ca2+-entry through L-type Ca2+ channels controls the profile of Ca2+-activated Cl- current in canine ventricular myocytes.
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