3rd, Closing scientific report for the project OTKA-PD101171

Title of the project:
Analysis of calcium-activated chloride current profiles in cardiomyocytes derived from various regions of the canine heart using the action potential voltage-clamp technique

Kalcium-függő kloridáram profilok vizsgálata a kutyaszív különböző régióiból származó szívizomsejteken akciós potenciál feszültség-clamp technikával

Principal investigator: Dr. Norbert Szentandrássy

SUMMARY OF THE PROPOSED PROJECT

The purpose of this project was to analyze calcium-activated chloride current ($I_{Cl(Ca)}$) profiles in cardiomyocytes derived from various regions of the canine heart using the action potential voltage-clamp (APVC) technique according to the title of the project OTKA-PD101171. In the schedule of the project, however, many other measurements were also planned for the three year period of the project. The major aims were the followings:

1. To explore the vertical (apico-basal) and transmural (subepicardial vs. subendocardial) gradient of $I_{Cl(Ca)}$ within the left ventricle of the canine heart as well as to study interventricular (left vs. right) differences of $I_{Cl(Ca)}$ using isolated canine cardiomyocytes. The regional differences were planned to be tested using several electrophysiological techniques including conventional sharp microelectrode recording and several types of voltage-clamp measurements including the probably most physiological one, the APVC.
2. To test the rate-dependent behavior of $I_{Cl(Ca)}$ during the time-course of the cardiac action potential (AP) using APVC. We wished to evaluate the effect of chloride channel blockers on duration of AP (APD) with conventional sharp microelectrode recording as well.
3. To evaluate the calcium-dependence of $I_{Cl(Ca)}$ in various experimental conditions.

In order to achieve these goals several types of experimental procedures were planned to use, including (1) isolation of canine cardiac myocytes from various ventricular regions, (2) measuring ion currents using various solutions having different intracellular free calcium concentrations ($[Ca^{2+}]$) with conventional voltage-clamp as well as with APVC and (3) recording APs with conventional sharp microelectrode technique.

RESULTS OF THE ACCOMPLISHED PROJECT

Experiments were performed using conventional microelectrode, whole-cell patch-clamp and action potential (AP) voltage-clamp techniques on isolated canine ventricular myocytes. Intracellular calcium transients were recorded with FURA-2 fluorescent dye with either field stimulation or together with AP recordings. 0.2 mM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and 0.5 mM anthracene-9-carboxylic acid (9-AC) have been used to study $I_{Cl(Ca)}$. All functional experiments were done at 37 ^{o}C degrees. Western blot and immunocytochemistry were also performed to detect the presence and localization of TMEM16A and Bestrophin-3 in canine ventricular myocytes. These proteins nowadays are often mentioned as promising candidates for the ion channel mediating $I_{Cl(Ca)}$.

Conventional AP measurements performed with 0.2 mM DIDS showed an increase of APD in a reverse-rate dependent manner. DIDS drastically reduced phase 1 and elevated the plateau potential of the APs in the six studied cells. Moreover, it turned out that the phase 0, the depolarization was also greatly reduced by DIDS suggesting a block of sodium current. Therefore it turned out that DIDS is not the most suitable drug to inhibit $I_{Cl(Ca)}$ during AP measurement. We used then 0.5 mM anthracene-9-carboxylic acid (9-AC) which had no effect on phase 0 and produced similar effects to those of DIDS regarding the other parameters of the AP. None of the two drugs affected resting membrane potential and both exerted reversible effects.

Under voltage-clamp conditions $I_{Cl(Ca)}$ (measured as DIDS sensitive current in eight cells without buffering $[Ca^{2+}]$ in the pipette) had a bell-shaped current-voltage relationship. The DIDS sensitive current activated rapidly (usually within 10-15 ms) and its decay was also fast as it reached its steady-state level within 12-18 ms. $I_{Cl(Ca)}$ measured as 9-AC-sensitive current under voltage-clamp was identical to that of DIDS-sensitive one not only in current density and kinetics but also in calcium-dependence as the application of 10 mM BAPTA in the pipette solution could abolish $I_{Cl(Ca)}$. The DIDS sensitive current density was approximately 25% smaller in the presence of 10 mM EGTA compared to that in the absence of any calcium buffer.

Regarding calcium-dependence of $I_{Cl(Ca)}$ we measured progressively higher peak amplitudes of $I_{Cl(Ca)}$ with higher stimulatory frequencies (shorter interpulse intervals) according to our expectations (n=6).

We also reported strong rate-dependence of the baseline, the peak value as well as the amplitude of field-stimulation induced calcium transients. Moreover, there was a strong correlation (r^2=0.996) between the amplitudes of $I_{Cl(Ca)}$ and the peak values of these calcium transients.
Then we studied the concentration-dependent actions of 9-AC on the shape of the AP with conventional AP measurements. It turned out that the actions of 9-AC were dose-dependent and 1 mM 9-AC produced no further changes when applied after 0.5 mM 9-AC. The half-maximal effects of 9-AC on APD and phase 1 were approximately 0.16 mM with Hill coefficients around 2.
To exclude the possibility that 9-AC increases APD and increases the plateau potential due to altering other ion channels, we tested the major ion currents shaping the cardiac AP ($I_{Ca,L}$, I_{Kr}, I_{Ks} and I_{K1}). None of these currents were altered by 0.5 mM 9-AC meaning, together with the previous results, that 9-AC applied in 0.5 mM is more suitable to study $I_{Cl(Ca)}$ in AP measurements than DIDS but the two inhibitors are equally good in those voltage-clamp studies where sodium current is blocked or kept inactive.
Most of these results were published recently [**1**].
In later experiments therefore 0.5 mM 9-AC was used to study $I_{Cl(Ca)}$.
Regarding the actions of the inhibitor on AP we observed region-dependence. At 1 Hz stimulation frequency 9-AC caused 24.34±3.26 ms and 3.36±1.05 mV increase in APD and plateau potential, respectively in 16 midmyocardial cell. Similar effects were seen in 7 subendocardial cells, where 9-AC caused 12.38±2.14 ms and 1.73±1.08 mV increases in APD and plateau potential, respectively but in 7 subepicardial cells both APD and plateau potentials were reduced by 20.04±3.58 ms and 2.00±1.10 mV, respectively. Moreover, 4-AP pretreatment of subepicardial cells, to reduce I_{to1} current and make their AP similar to subendocardial ones, prevented the reduction of both APD and plateau potential induced by 9-AC and those parameters changed similarly to that observed in subendocardial and midmyocardial cells.
Regarding rate-dependence, 9-AC acted in a reverse-rate dependent manner in subendocardial and midmyocardial cells where the prolongation of AP was greater at slow stimulation rate. In subepicardial cells the shortening of APs was also more pronounced towards slower stimulation rates between 500 and 5000 ms.
Apico-basal differences on 9-AC-induced AP effects were also observed within the left ventricular midmyocardium. Although the reduction of phase 1 was similar in all 28 cells but the increase of APD was significantly greater in the 13 basal cells (30.29±3.62 ms at 1 Hz) compared to what have been seen in the 15 apical cells (17.98±2.86 ms at 1 Hz). 9-AC increased APD again in a reverse-rate dependent manner in all 28 cells. There was an apico-basal difference in the elevation of plateau potentials induced by 9-AC as regardless of the stimulation rate the elevation was always greater in basal cells.
Regarding APVC experiments, $I_{Cl(Ca)}$ was measured as 9-AC sensitive current and showed an early, narrow outward current peak having a value of approximately 1.5 A/F at 1 Hz stimulation rate and without buffering [Ca^{2+}] in the pipette solution. The peak of this outward current corresponded to phase 1 of the command AP. Towards the end of the command AP (at around the time of 85-90% of repolarization) a late, inward peak with much smaller amplitude (approx. -0.2 A/F) was present on the 9-AC sensitive current.
These two peaks were the representation of $I_{Cl(Ca)}$ during the AP as their amplitude were dependent on intracellular [Ca^{2+}] as demonstrated by the increase of [Ca^{2+}]. This was achieved by the application of 10 nM isoproterenol (ISO) (outward and inward peaks of 3.10±0.21 and -0.36±0.11 A/F, respectively, n=6) and 20 nM BayK8644 (outward and inward peaks of 2.86±0.15 and -0.30±0.03 A/F, respectively, n=8). On the contrary, reducing intracellular [Ca^{2+}] by calcium chelators in the pipette solution reduced the current (10 mM EGTA to 1.17±0.09 and -0.13±0.02 A/F whereas 10 mM BAPTA to 0.13±0.10 and -0.08±0.02 A/F).
Further support for the fact that 9-AC sensitive is indeed $I_{Cl(Ca)}$ is the chloride sensitivity of the two peaks as the application of low chloride concentration in the pipette solution shifted the reversal potential of the current to more negative membrane potential values resulting in the increase of the early outward peak and the reduction of the late, inward peak. Plotting the phase-plane trajectory of

$I_{Cl(Ca)}$ showed that the outward current is active only in a narrow voltage-range of the AP which is exactly the one corresponding to phase 1.
The rate-dependence of the 9-AC sensitive current measured with APVC was similar as that of the voltage-clamp data, namely the current peaks (both outward and inward ones) were larger at 2 Hz stimulation rate that those recorded at 1 Hz.
We have also tested the difference of 9-AC sensitive current measured during actual APs among cells isolated from various layers with APVC technique. The early, narrow outward current peak was 1.77±0.08, 1.53±0.11 and 1.35±0.12 A/F in subepicardial, midmyocardial and subendocardial cells (n=5, 15, 6), respectively. Although subepicardial cells possessed a significantly larger outward current peak compared to the other cells, the late, inward peaks was not significantly different among these cell populations having an average value of approximately -0.17 A/F.
Then the shape of the command AP on the amplitude of 9-AC sensitive current was evaluated by applying both a subepicardial and a subendocardial type of AP to both subepicardial and subendocardial cells. These experiments confirmed the importance of the configuration of the AP in setting the size of the outward peak of $I_{Cl(Ca)}$ as regardless of the origin of the cell subepicardial command AP always generated larger currents than subendocardial ones.
This importance is further supported by the fact that current densities and voltage-dependence of 9-AC-sensitive current measured during voltage-clamp was identical regardless of the origin of the cells as no difference was observed either in transmural or apico-basal direction.
The calcium-dependence of $I_{Cl(Ca)}$ was further tested as 9-AC-sensitive current was measured with various pipette solutions designed to mimic the systolic and diastolic values of bulk intracellular [Ca^{2+}] (0.1 and 1.1 μM, respectively). The current measured at +60 mV was significantly larger when 1.1 μM was the calculated free [Ca^{2+}] in the pipette solution than in the case of 0.1 μM. This latter low concentration hardly evoked any current as the current density was only slightly higher than that measured when 10 mM BAPTA was used without any added calcium in the pipette solution. Even 1.1 μM free calcium evoked much smaller current (0.31±0.06 A/F) than that measured without buffering [Ca^{2+}] (2.44±0.28 A/F). The calcium-dependence of the current was further supported when we measured $I_{Cl(Ca)}$ in the presence of nisoldipine. Here, $I_{Ca,L}$ as well as $I_{Cl(Ca)}$ were reduced to nearly zero supporting the fact that Ca^{2+} influx through L-type calcium channels is essential for the activation of $I_{Cl(Ca)}$.
We also tested the importance of calcium induced calcium release through ryanodine receptors in the activation of $I_{Cl(Ca)}$. In the presence of 10 μM ryanodine (shown to almost completely abolish both cell shortening and calcium transients) the further reduction of phase 1 induced by 9-AC was still detectable during sharp microelectrode AP measurements. In APVC recording, the values of both the outward and inward peaks were significantly smaller in the presence of ryanodine than those in control. These data supports our hypothesis that calcium induced calcium release is not essential for the activation of $I_{Cl(Ca)}$ and is mainly influenced by the subsarcolemmal [Ca^{2+}]. This is also supported by our finding that not only the fast buffer BAPTA but also the slow buffer EGTA completely abolishes calcium transients but only BAPTA is effective enough to buffer subsarcolemmal calcium and therefore to abolish $I_{Cl(Ca)}$.
Regarding the expression and localization of the potential channel proteins of $I_{Cl(Ca)}$ we detected the presence of TMEM16A as well as Bestrophin-3 on the cell membrane and in striations (likely t-tubules) of isolated cardiomyocytes in close proximity of Cav1.2 and each other. Comparing cells derived from various regions, Western blot data revealed identical expression of TMEM16A in apical and basal cells of the left ventricular midmyocardium.
To evaluate the potential pro- and antiarrhythmic role of $I_{Cl(Ca)}$ sharp microelectrode AP measurements were conducted. Out of the 28 studied cells only two showed early afterdepolarizations (EAD, an arrhythmogenic event) in the presence of 9-AC at slow stimulation rates and no delayed afterdepolarizations were noted. If 9-AC was applied in the presence of ISO, more than 35% of the same cell population generated EADs.
During our experiments we tested the effect of 9-AC on short term variability of APD (SV) by recording 50 consecutive APs and calculated SV as it follows: SV = [Σ (| APD; i+1 minus APD; i |)] / [50 * √2]. The increase of SV is a good predictor of cardiac arrhythmias. In the presence of 9-AC,

SV was higher being 3.08±0.22 ms than in control (2.33±0.14 ms). Adding ISO to the same 24 cells in the presence of 9-AC SV was reduced to 1.83±0.18 ms which was significantly lower than that in control. However, this value is still significantly higher than SV in ISO alone (1.43±0.11 ms, n=13). The reduction in SV induced by ISO was significantly less in the presence of 9-AC compared to that seen without 9-AC.
Most of these results were published as citable abstracts of various conferences [**2-5**].
We are currently writing two articles from our unpublished results and plan to submit them for publication soon.

DETAILED DIFFERENCES BETWEEN THE PROPOSED AND THE ACCOMPLISHED PROJECT

The following experiments initially planned to be carried out were actually not done in the project:
1. Perforated patch-clamp measurements, but we carried out the examination of the rate-dependence of $I_{Cl(Ca)}$ using whole-cell voltage-clamp using a pipette solution without calcium buffering.
2. The evaluation of the interventricular (left versus right ventricle) differences of $I_{Cl(Ca)}$.

The following experiments were done in a less complete way in the project:
1. The rate-dependence of the action of $I_{Cl(Ca)}$ inhibition on sharp microelectrode recorded AP was studied with the application of only half of the planned stimulation rates, which however does not reduce the value of our finding according to us.
2. The rate-dependence of $I_{Cl(Ca)}$ profiles studied by APVC was restricted to the comparison of 2 Hz and 1 Hz stimulation.

We carried out, however, many additional experiments which were not planned to be done in the proposed project.
These include (1) the dose-dependent evaluation of the actions of 9-AC on AP, (2) determination of the half effective concentration of 9-AC regarding the increase of APD and the reduction of phase 1, (3) evaluation of the specificity of 9-AC by testing its potential effects on four ion currents including $I_{Ca,L}$, I_{Kr}, I_{Ks} and I_{K1}, (4) exploration of the source of calcium for the activation of $I_{Cl(Ca)}$ (influx via L-type calcium channels and/or calcium induced calcium release through ryanodine receptors), (5) demonstration of the chloride sensitivity of $I_{Cl(Ca)}$ profiles with APVC (6) determination of the expression and the localization of TMEM16A and Bestrophin-3 proteins and (7) demonstration of $I_{Cl(Ca)}$ inhibition on EAD formation and short term variability of APD in control and in the presence of ISO.

PREDICTIONS OF THE PROPOSED PROJECT AND THEIR REALIZATION

Comparing our actual results with our predictions made at the time of the submission of the proposed project we can state the followings:
We correctly predicted (1) the lack of regional differences of the densities of $I_{Cl(Ca)}$ measured with voltage-clamp, (2) the difference of $I_{Cl(Ca)}$ profiles between subepi- and subendocardial cells during APVC, (3) the calcium- and also (4) the rate-dependence of $I_{Cl(Ca)}$ amplitude with both voltage-clamp and APVC.
Our prediction about the profile of $I_{Cl(Ca)}$ to be more complicated was wrong as only the value of the first, early outward peak was higher in subepicardial cells. Our hope to find an intervention in the form of chloride channel inhibition, which does not show reverse rate-dependence on the influence on APD faded. Moreover, inhibition of $I_{Cl(Ca)}$ proved to be actually proarrhythmic.

SUMMARY OF MAJOR FINDINGS OF THE PROJECT

1. DIDS in only suitable to study $I_{Cl(Ca)}$ when sodium channel inhibition by the drug is not problematic.
2. 0.5 mM 9-AC is specific and sufficiently high dose to examine the role of $I_{Cl(Ca)}$.
3. Only BAPTA, but not EGTA is effective in buffering fast changes in $[Ca^{2+}]$ in the subsarcolemmal space.
4. Only high, subsarcolemmal $[Ca^{2+}]$ increase activates $I_{Cl(Ca)}$, whole-cell systolic $[Ca^{2+}]$ is not enough.
5. Blockade of $I_{Ca,L}$ eliminates $I_{Cl(Ca)}$, whereas higher frequencies of stimulation as well as isoproterenol and BayK8644 increase the current.
6. Ryanodine decreases $I_{Cl(Ca)}$, but not abolishes it.
7. Altering chloride concentration in the pipette solution modified the profile of $I_{Cl(Ca)}$ confirming chloride as its charge carrier.
8. TMEM16A and Bestrophin-3 are expressed on canine ventricular myocytes.
9. TMEM16A and Bestrophin-3 are present on t-tubules and highly likely co-localize with not only each other but also with Cav1.2 (L-type calcium channel).
10. $I_{Cl(Ca)}$ diminishes the extent of transmural and apico-basal temporal differences in repolarization.
11. Larger $I_{Cl(Ca)}$ can be measured under subepicardial AP, due to higher Ca^{2+} entry through Cav1.2.
12. The expression level of TMEM16A is equal in apical and basal cells of the left ventricular midmyocardium just as the amplitudes of $I_{Cl(Ca)}$ measured with rectangular voltage pulses.
13. $I_{Cl(Ca)}$ reduces the extent of short term variability of APD.
14. $I_{Cl(Ca)}$ has a protective role against EAD formation.
15. $I_{Cl(Ca)}$ contributes to shortening of AP in response to β-adrenergic stimulation.

CONCLUSION OF THE PROJECT

Our results highlight that a blockade of $I_{Cl(Ca)}$ could actually be harmful (especially at bradycardia) by not only due to the increase in dispersion of repolarization but also by generating EADs. Moreover, the value of SV was also higher in 9-AC suggesting that $I_{Cl(Ca)}$ could be a useful ion current and its presence could be regarded as antiarrhythmic.

POTENTIAL BENEFITS OF THE PROJECT

This project can fully be considered as basic science. According to our results, potential reduction in regional AP heterogeneity can be achieved by chloride channel activators. These drugs would also reduce short term variability of APD thereby could be beneficial in prevention of arrhythmias. Unfortunately, on the contrary to this and according to the literature the development of delayed afterdepolarizations can be the consequence of $I_{Cl(Ca)}$ activation, therefore care must be taken especially in such disease states where calcium overload can occur.

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Out of the total 11, 7 were presented on international conferences.

1. 36th Meeting of the European Working Group on Cardiac Cellular Electrophysiology, Nantes, 2012
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Antiarrhythmic properties of calcium-activated chloride current in canine left ventricular myocardium

PUBLISHED IN EXTENSO PUBLICATION SUPPORTED BY THE PROJECT IN THE TOPIC OF THE PROJECT:

Váczi K, Hegyi B, Ruzsnavszky F, Kistamás K, Horváth B, Bányász T, Nánási PP, Szentandrássy N, Magyar J
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[IF=2.36]

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13 articles with the combined impact factor of 44.603

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