Electrophysiological properties and pharmacological modulation of several transmembrane ion currents in mammalian hearts

Summary of PhD Thesis

Amir Mohammad Geramipour, *DPharm*

Supervisor
Norbert Jost, *PhD*

Department of Pharmacology and Pharmacotherapy
Faculty of Medicine, University of Szeged

Doctoral School of Multidisciplinary Medicine
University of Szeged

Szeged, Hungary

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1. List of publication related to the subject of the PhD thesis

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2. Abstract.

Cardiovascular diseases and in particular cardiac arrhythmias as ventricular fibrillation have a leading role in mortality in the developed countries. Accordingly, cardiac arrhythmias represent a major area of cardiovascular research. Drug therapy has traditionally been the major type of treatment for both ventricular and supraventricular arrhythmias. In spite of the important advances in cardiology, the pharmacological treatment of cardiac arrhythmias remained empiric to a large extent because of our incomplete understanding the mechanisms by which antiarrhythmic drugs prevent/suppress, or unfortunately even induce arrhythmias. Therefore, in order to develop new more effective agents with less proarrhythmic potency, it is important to understand the mechanism of action of antiarrhythmic drugs at the organ, tissue, cellular and also subcellular levels. Based on the promising results of recent cellular pathophysiological and pharmacological investigations in the present PhD thesis, we have focused our research especially on pharmacological modulation of repolarizing potassium and sodium-calcium exchanger (NCX) currents as key elements in generating arrhythmias.

The aims of the present study were:

a) To analyse the contribution of different auxiliary proteins to the altered expression of genes for Kir2.x ion channels in dilated cardiomyopathy.

b) To investigate and compare the effects of several newly synthetized NCX blockers on several mammalian hearts. Selection of a lead NCX blocker compound for further analysis.

c) To investigate the electrophysiological effects of the lead compound GYKB-6635, a newly synthetized specific NCX inhibitor, on the NCX, L-type Ca^{2+} and several potassium currents and on the triggered arrhythmias (formation of delayed afterdepolarizations).

Our results demonstrate:

1) The endogenous Kir2.x channels associate with SAP97 forming signalling complexes. In DCM, the levels of Kir2.1 and Kir2.3 were upregulated but those of Kir2.2 channels were down-regulated. These adaptations could offer a new aspect for the explanation of the generally observed physiological and molecular alterations found in DCM. The SAP97 and Kir2.x ion channels may be novel target molecules in the diagnosis and effective treatment of cardiomyopathy.
2) We have demonstrated in vitro the potential inhibitory NCX blocking effect and of several carbocyclic nucleoside analogues (CNA) having different structures than from already known selective NCX blockers.
3) GYKB-6635 is the first compound that inhibits forward and reverse mode of the NCX current at submicromolar concentrations, and does not affect any other important transmembrane mechanisms involved in Ca^{2+}-homeostasis and cardiac repolarization. In addition, GYKB-6635 compound proved to be effective against DAD related arrhythmias, since in isolated Langendorff perfused heart experiments prevented disturbances of the heart rhythm in ouabain induced arrhythmias in guinea pigs.
4) In conclusion, in the present study we describe a new and highly selective NCX inhibitor compound that may be suitable to test whether NCX blockade offers beneficial antiarrhythmic effects.

3. Introduction

Cardiovascular diseases, and in particular, cardiac arrhythmias, such as ventricular fibrillation have a leading role in mortality in developed countries. The most serious ventricular arrhythmia – ventricular fibrillation – causes the death of more than 3.000.000 people all over the world and 300.000 – 350.000 people in the USA and Europe annually, which statistically means that one person dies every minute on each continent. In the majority of the cases sudden cardiac death occurs when victims are not in hospital, consequently, survival probability is very low. Most frequently (50%) the background of the on-the-spot diagnosed circulation collapse is ventricular tachycardia /fibrillation/. Sudden cardiac death (SCD) is often the very first sign of the symptom-free cardiovascular disease. SCD is a complex national health problem affecting families and having significant social and economic consequences, since usually it is the head of the family, a seemingly healthy man, who dies tragically. Accordingly, cardiac arrhythmias represent a major area of cardiovascular research. One of the main goals of pharmacological research is to develop a safe ventricular antiarrhythmic drug that can be applied either in acute cases or for treating postinfarction patients.

In the past, drug treatment of cardiac arrhythmias has proven difficult, both because of inadequate effectiveness and a risk of serious complications. In spite of the important advances in cardiology, the pharmacological treatment of cardiac arrhythmias remained empiric to a large extent because of our incomplete understanding of either physiological and pathophysiological processes underlying the cardiac rhythm disturbances and the mechanisms by which antiarrhythmic drugs prevent, suppress, and in some cases also induce, arrhythmias. Therefore, in order to develop new more effective agents with less proarrhythmic potency, it is important to understand the mechanism of action of antiarrhythmic drugs at the organ, tissue, cellular and also subcellular levels.

Dilated cardiomyopathy (DCM) is a myocardial disorder characterized by left ventricular dilation and systolic dysfunction often leading to progressive heart failure, arrhythmias and premature death. DCM is associated with increased APD, decreased resting membrane potential (RMP), and the whole-cell current slope conductance in cells of DCM is smaller than that for donor or ischemic cardiomyopathy. As a chronic multifactorial disease, DCM is likely to affect multiple clusters of genes. Marked alterations were observed in the

characteristics of the inward rectifier potassium current (I_{K1}), but not of the sodium current (I_{Na}) in ventricular myocytes of DCM patients as compared to donors.. The I_{K1} current plays a major role for maintaining the cellular resting membrane potential and it is involved in the consequences of DCM such as in the arrhythmogenesis of coronary artery disease and ventricular arrhythmias with sudden cardiac death. However, the molecular mechanisms underlying these alterations of I_{K1} are still largely unknown.

One arrhythmogenic factor that can result in ventricular arrhythmias occurring in myocardial ischaemia or poisoning with digitalis is delayed afterdepolarization (DAD), which arises in heart muscle cells following Ca^{2+} overload. Reducing the incidence of these trigger mechanism, the delayed afterdepolarization (DAD) or their pharmacological blockade would be extremely desirable from a clinical point of view.

Among several other currents and transporters, maintenance of the Ca^{2+} homeostasis in the myocardium is mainly regulated by the sodium-calcium exchanger (NCX). It is known that NCX, at the forward mode, extrudes Ca^{2+} from the cell to the extracellular space during diastole, at relatively low free cytoplasmic Ca^{2+} concentration and negative transmembrane potential. Since the extrusion of one Ca^{2+} is coupled with 3 Na^{+} entering the cell, during the forward mode of the NCX net inward current is carried, which can cause substantial depolarization leading to early (EAD) and delayed (DAD) after-depolarizations, especially when intracellular Ca^{2+} is elevated. Neither our current knowledge on NCX structure/function relationship is complete, nor the selectivity of current NCX modulators are satisfactory especially in conditions where potassium conductance is decreased, such as heart failure Therefore, one may speculate that specific blockers of NCX could be potential antiarrhythmics in dysrhythmias related to Ca^{2+} overload. This hypothesis could not be directly tested since the available NCX inhibitors. This unmet need provides a rationale for pharmacologists and medicinal chemists to get more involved in NCX research by designing and developing novel compounds to modulate NCX activity.

Therefore, in the present study carbocyclic nucleoside analogues (CNA) as potential novel selective inhibitors of NCX current and their effect on DAD related and ischemia--reperfusion (IR) induced cardiac arrhythmias are investigated. A particular attention was played to investigate the effects of the CNA derivate and highly selective novel NCX inhibitor GYKB-6635 [(4-amino-1-[((1*R*,2*S*,3*S*,5*R*)-2,3-dihydroxy-6,6-dimethylbicyclo[3.1.1] heptan-2-yl)methyl]pyrim idin-2(1*H*)-one) on the NCX current, cardiac ventricular action potentials and to determine its antiarrhythmic effects.

4. Major specific experimental goals

The main goals of my PhD work was to investigate: *(i)* the remodelling effect of one specific cardiac disease on the repolarization; *(ii)* the pharmacological modulation of the cardiac arrhythmias by affecting a novel pharmacological target. Thereby the specific goals were as follows:

a) To analyse the contribution of different auxiliary proteins to the altered expression of genes for Kir2.x ion channels in dilated cardiomyopathy.

b) To investigate and compare the effects of several newly synthetized NCX blockers on several mammalian hearts. Selection of a lead NCX blocker compound for further analysis.

c) To investigate the electrophysiological effects of the lead compound GYKB-6635, a newly synthetized specific NCX inhibitor, on the NCX, L-type Ca^{2+} and several potassium currents and on the triggered arrhythmias (formation of delayed afterdepolarizations).

5. Methods

Experiments were carried out in ventricular myocytes enzymatically isolated from dog and rabbit hearts and from undiseased human cardiac ventricular preparations. The protocols used on guinea pig and dog heart preparations were approved by the Review Board of the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development, Hungary (XIII./1211/2012). The experimental protocols used on human myocytes complied with the Declaration of World Medical Association proclaimed in Helsinki and were approved by the University of Szeged and National Scientific and Research Ethical Review Boards (No. 51-57/1997 OEj and 4991-0/2010-1018EKU (339/PI/010.)).

The *in vitro* electrophysiological (standard microelectrode and patch-clamp techniques) measurements were performed in dog and human cardiac preparations. Langendorff perfused guinea hearts were used for ouabain induced arrhythmia studies. Human Kir2.x protein level and mRNA level expressions and immunofluorescence investigations were performed by applying several molecular cardiology methods including Western-blot and qPCR techniques and confocal microscopy, respectively.

6. Results

6.1. The investigation of the expression of genes for Kir ion channel isoforms in dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is a multi-factorial disease characterized by left ventricular dilation that is associated with systolic dysfunction and increased action potential duration. The Kir2.x K^+ channels (encoded by *KCNJ* genes) regulate the inward rectifier current (I_{K1}) contributing to the final repolarization in cardiac muscle. Here, we describe the transitions in the gene expression profiles of four *KCNJs*, from non-diseased to dilated cardiomyopathic human hearts.

Cardiac left ventricular samples were obtained from 12 hearts of DCM patients and compared with those of 12 undiseased donors. We observed marked changes in the relative mRNA levels of I_{K1}-related genes in the ventricle of DCM patients as compared to controls. Thus Kir2.1 and SUR1 mRNA levels increased, while those for Kir6.1 α-subunit and SUR2 β-subunit were declined relative to undiseased controls. Relative mRNA level for Kir3.4 was also reduced in patients, whereas the mRNA levels of Kir3.1 and Kir6.2 did not differ between hearts of control and DCM patients.

Next we performed immunoblotting analysis of protein samples matching the RNA sources to reveal if differences in steady state mRNA levels were manifested in protein content. As expected, anti-human Kir2.1, Kir2.2, and Kir2.3 antibodies recognized bands of 55 kDa, 45 kDa and 57 kDa, respectively. Kir2.1 protein expression was increased in the ventricles of DCM hearts confirming thus the mRNA data. Immunoblots were reprobed with anti-GAPDH and/or α-actin antiserum and the intensity of immunosignals was then quantified by image analysis software relative to GAPDH or α-actin internal controls. The normalized protein amount of Kir2.1 isoform was higher (with about 70 %) showing some individual variability, while the Kir2.2 isoform was lower (by about 29 %) in DCM compared to control hearts. The Kir2.3 protein content, however, did not differ considerably between donor and DCM ventricular tissue samples.

Taken all these data together we may conclude that, qRT-PCR analysis confirmed that mRNA expression for Kir2.1 (*KCNJ2),* Kir2.3 (*KCNJ4)* and Kir2.4 (*KCNJ14)* coding genes significantly increased (2.26-fold, 1.94-fold and 1.6-fold, respectively), while mRNA level for Kir2.2 (*KCNJ12)* was reduced to 66% in DCM ventricle *versus* control. In agreement with

RNA data, Western analysis revealed increased Kir2.1 and decreased Kir2.2 protein levels in DCM patients, but the Kir2.3 level was not markedly altered as compared to control.

Considering that SAP97 can associate with Kir2.2 and Kir2.3 isoforms in the heart samples, we observed significant changes in Kir2.1 and Kir2.2 mRNA and protein levels, it was interesting to check whether mRNA level for SAP97 regulatory protein has also changed in DCM. Gene expression for SAP97 was validated by qRT-PCR. The SAP97 mRNA level decreased significantly in DCM patients. Furthermore, we observed robust age-dependent changes in SAP97 mRNA expression even in donor hearts, when we compared 31 undiseased and 17 DCM samples. SAP97 mRNA level decreased about 50% at age of 40 years or higher. SAP97 mRNA levels were high at ages between 12-40 years in control cardiac ventricles and decreased by 52 % in DCM patients. After 40 years of age, however, the differences in the SAP97 mRNA levels diminished between control and DCM hearts.

Based on the robust changes in Kir2.x and SAP97 mRNA expression in DCM *versus* normal heart, we hypothesized that SAP97 binding to Kir2x isoforms and distribution of their complexes might have changed in cardiomyocytes of DCM heart. To address the potential differences in the distribution of SAP97-Kir2.x complexes in DCM, we used indirect immunofluorescence. Double immunofluorescence showed the colocalization of SAP97 with type of Kir2.1 or Kir2.2 isoforms in cardiomyocytes of ventricular tissues in both control and DCM hearts. Whereas the distribution of Kir2.1 and SAP97 partially overlapped in the intercalated discs of control sections, their colocalization was more obvious in failing human ventricle as judged by the number and intensity of merged yellow spots (data not shown).

During the last decade a few publications have suggested from the altered action potential (AP) shape in DCM that the properties of I_{K1} current could be changed (Koumi *et al*. 1995). Therefore, we studied the I_{K1} current in human cardiomyocytes derived from the heart of undiseased donors and DCM patients. We measured I_{K1} by whole cell patch-clamp technique in the isolated ventricular myocytes. At -60 mV I_{K1} density was moderately lower in the undiseased donor cardiomyocytes than in DCM human ventricular myocytes. Steady-state inward I_{K1} density was reduced in cells isolated from DCM ventricle compared with undiseased myocardium.

6.2. The investigation of several newly synthetized CNA compounds as novel selective NCX inhibitors. The selection of GYKB-6635 as lead compound

In the present study carbocyclic nucleoside analogues (CNA) as potential novel selective inhibitors of NCX current and their effect on DAD related and ischemia-reperfusion (IR) induced cardiac arrhythmias are investigated in mammalian hearts. The structures of these CNA compounds basically differ from the already known selective NCX blockers, like KB-R7943 and SEA-0400 or from ORM-10103.

The following compounds were synthetized and investigated: GYKB-6635; GYKB--6704; GYKB-6707. NCX current was measured by applying the whole cell patch-clamp technique. Current was measured as a Ni^{2+} sensitive current using voltage ramp waveforms for the command potential. The effect of various concentrations of the compounds on outward NCX current (reverse mode) was calculated at 20 mV, and on inward current (forward mode) was determined at -80 mV. Based on these results we conclude that GYKB-6635, GYKB--6704 and GYKB-6707 effectively inhibit the NCX current at micromolar concentration ranges. The most potent compound seems to be GYKB-6635, therefore this compound has been selected for further analysis described in the chapter 3.3.

6.3. Investigation of the cellular electrophysiological and antiarrhythmic effects of GYKB-6635 in mammalian hearts

The aim on the present investigations was the systematic investigation of the carbocyclic nucleoside analogue compound GYKB-6635, as lead highly selective NCX blocker compound. The potential antiarrhythmic potency of GYKB-6635 compound was also investigated in detail. The results of the experiments showed that 1 μM GYKB-6635 reduced by 57 % the reverse NCX current (from 52.5 ±18.5 pA in controls to 22.4 ±11.9 pA after drug administration, $n=4$, $p<0.05$), while the forward current was reduced by 58 % (from -52.7 ±21 pA in controls to -21.8 ±12.3 pA after drug administration, $n=4$, $p<0.05$).

This blocking effect was quite selective, because it was shown that, GYKB-6635 even at high (10 μM) concentration did not influence I_{CaL}, the fast inward sodium current (I_{Na}), and the four main repolarizing currents, I_{to}, I_{K1}, I_{Kr} and I_{Ks}. The selective blocking effect of the GYKB-6635 compound proved to be effective against DAD related arrhythmias, since in isolated Langendorff perfused heart experiments prevented disturbances of the heart rhythm in ouabain induced arrhythmias in guinea pigs.

7. Conclusions and potential significance

The conclusions and main findings of the present thesis are as follows:

1) The endogenous Kir2.x channels associate with SAP97 forming signalling complexes. The Kir2.1 strongly binds SAP97, and they show co-localization near the T-tubules. In undiseased adult ventricle, KCNJ2, KCNJ12 and KCNJ4 (Kir2.1-2.3) genes were expressed at high level; while the expression of KCNJ14 (Kir2.4) gene was low. In DCM, the levels of Kir2.1 and Kir2.3 were upregulated but those of Kir2.2 channels were down-regulated. These adaptations could offer a new aspect for the explanation of the generally observed physiological and molecular alterations found in DCM. The SAP97 and Kir ion channels may be novel target molecules in the diagnosis and effective treatment of cardiomyopathy.

2) We have demonstrated *in vitro* the potential inhibitory NCX blocking effect and of several carbocyclic nucleoside analogues (CNA). The structures of these CNA compounds basically differ from the already known selective NCX blockers, like KB-R7943, SEA0400 and ORM-10103.

3) GYKB-6635 is the first compound that inhibits forward and reverse mode of the NCX current at submicromolar concentrations, and does not affect any other important transmembrane mechanisms involved in Ca^{2+}-homeostasis and cardiac repolarization. In addition GYKB-6635 compound proved to be effective against DAD related arrhythmias, since in isolated Langendorff perfused heart experiments prevented disturbances of the heart rhythm in ouabain induced arrhythmias in guinea pigs.

4) We may concluded that GYKB-6653 is a new and highly selective NCX inhibitor compound that may be suitable to test whether NCX blockade offers beneficial antiarrhythmic effects. Further studies are needed using both *in vitro* and *in vivo* methods to elucidate the potential therapeutic targets and, in a wider sense, the possible beneficial effects of specific NCX inhibition.

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