SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Study of the regulation of Kv1.3 channels by sterols in *in vitro* and *ex vivo* systems

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Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Examination Committee**:	Zoltán Papp, MD, PhD, DSc
Members of the Examination Committee:	János Magyar, MD, PhD, DSc
	János Matkó, PhD, DSc

The Examination takes place at the Discussion Room (2.209-2.211) of the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen
13.10. 2016, 11 a.m.

Head of the **Defense Committee**:	Zoltán Papp, MD, PhD, DSc
Reviewers:	Barna Vásárhelyi, MD, PhD, DSc
	Beatrix Dienes, PhD
Members of the Defense Committee:	Zoltán Papp, MD, PhD, DSc
	János Magyar, MD, PhD, DSc
	János Matkó, PhD, DSc
	Barna Vásárhelyi, MD, PhD, DSc
	Beatrix Dienes, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
13.10. 2016, 13 p.m.

I. INTRODUCTION

I.1. Ion channels: general properties

Ion channels are pore-forming, integral membrane proteins allowing the transport of inorganic ions down their electrochemical gradient across the cell membrane. They have three characteristic features: gating, selective permeability and conductance. The term gating refers to a transition/conformational change between different functional (open, closed) and/or structural (open, closed, inactivated) states upon a specific stimulus. Ion channels can be classified by this special stimulus as voltage-, extracellular ligand, intracellular messenger and stretch-gated channels. Ion channels provide selective transport for a given ion (or group of ions). Based on their selectivity we distinguish highly selective, mildly selective and non-selective ion channels.

I.2. Structure and function of the voltage-gated Kv1.3 channel

Kv1.3 is a voltage-gated, homotetrameric channel formed by four, 523-amino-acid-residue-long, non-covalently bonded identical subunits. Every subunit composed of six transmembrane alfa-helices connected by intra and extracellular loops. Each subunit contain a 94-amino-acid-residue-long intracellular C-terminal and a 184-amino-acid-residue-long intracellular N-terminal. While the transmembrane segments are highly ordered, conservative regions, the flexible C-terminus can take up a random coil but not a rigid structure. Kv1.3 subunits can assembly with other Kv isoforms (Kv1.1, Kv1.2, Kv1.4, Kv1.5) to form functional channels. The subunit composition determines the structural, biophysical and pharmacological properties of the heteromers. The Kv1.3 ion channel was described as a transmembrane protein of the human T cells in 1984. Comprehensive proteomic studies demonstrated that the Kv1.3 also expressed by other immune cells (B cells, dendritic cells) and in the central nervous system and gastrointestinal tract. The most widely studied function of Kv1.3 is the regulation of calcium signaling during T cell activation by keeping the membrane potential at negative values. By the use of Kv1.3-selective antagonists lymphocyte proliferation, migration and IL-2, IFN-γ production can be inhibited. Due to the specific ion channel expression pattern of the activated effector memory (T_{EM}) cells, specific Kv1.3 antagonists can be novel drugs in the treatment of the autoimmune disorders (i.e. multiple sclerosis, rheumatoid arthritis, type 1 diabetes) where mainly T_{EM} cells are responsible for the inflammation and tissue distruction.

I.3. Cholesterol regulation of ion channels

In the last few years many studies have emphasized the role of cholesterol in the regulation of ion channels. Because of the structural and functional variability of the ion channels cholesterol has distinct influence on the different channels. The general effect of cholesterol is usually a dose-dependent decrease in the channel activity (open probability, unitary conductance), which was demonstrated for many K^+ and also Ca^{2+}, Na^+ channels. Contrary, TRP channels' activity is inhibited by the removal of cholesterol. Cholesterol can also modulate the kinetic and equilibrium parameters of the ion channels. Two main models explain the effects of cholesterol on ion channels' function. The first theory focuses on the direct and specific lipid-protein interactions: cholesterol is involved in the assembly of an annular lipid belt around the ion channel. The components of this lipid belt or shell can easily interact with the transmembrane helices of the ion channels which contain specific lipid receptors. The manipulation of the membrane cholesterol level can change the conformation of the ion channels via modify these specific lipid-protein interactions. Recent studies have demonstrated that not just the classical lipid-protein interfaces but also the intracellular C and N terminal can interact directly with cholesterol through cholesterol recognition sites. Several putative cholesterol recognition sequences have been identified, which predicts that a transmembrane protein is to interact with cholesterol. Among them the CRAC and CARC sequences were the first documented and they are considered the most significant. The CRAC (cholesterol recognition amino acid consensus motif) is based on a very simple algorithm: it begins with a branched non-polar amino acid (leucine or valine) followed by 1-5 arbitrary amino acids, then, through an aromatic tyrosine, 1-5 other optional amino acids precede(s) a positively charged amino acid (lysine, arginine) that closes the sequence. The CARC sequence is the 'inverse' of the CRAC. It starts with a positive amino acid and, along with tyrosine, the closing aromatic amino acid can be phenylalanine, too. Due to the simplicity of the algorithm, the sterol sensitivity of proteins with CRAC/CARC sequence should be treated with careful skepticism, it only may serve as a predictive tool. However, we cannot ignore the results according to which a simple point mutation or deletion of these motifs could totally alter the cholesterol sensitivity of wild-type ion channels.

From another point of view, the cholesterol can influence the ion channels' operation via changing the physical parameters of the cell membrane. Due to the difference in the thickness of the hydrophobic parts of the transmembrane proteins and the lipid bilayers, the protein perturbs the surrounding lipids, thus changing the thickness and the order of the

bilayer. The bilayer adjusts its local thickness equals to that of the hydrophobic region of the transmembrane protein. During the conformational change, such as activation of an ion channel, the proteins deform the surrounding lipid bilayer: the stronger the resistance of the membrane is, the more energy is needed for this process to occur. The modification of the physical parameters of the lipid environment can change the molecular movements via modifying the energy required for the process. The cholesterol molecules affect the fluidity, thickness and permeability of the membrane along with the lateral diffusion of the membrane protein, and thus they can influence kinetic and equilibrium parameters of the ion channels' conformational change. These two models do not exclude each other, but the influence of the individual mechanisms (direct vs. indirect sterol effect) can differ from a channel to another. The separation of two effects for a particular channel provides researchers with a challenge.

A possible method is based on the investigation of cholesterol analogues whose impact on the membrane is similar to that of the cholesterol but cannot replace it in the specific sterol-protein interaction. The epicholesterol and the ent-cholesterol are two chiral cholesterol analogues that, with minor divergence, meet the abovementioned criteria. While the cholesterol decreases the Kir.2.1 channel's current density, epicholesterol increases it, indicating a specific lipid-protein interaction. Stereo-specificity of cholesterol analogues have been reported in the case of BK and TRPV1 channels, while nAchR or VRAC channels didn't not show any difference in the impact of the optical isomers and the cholesterol.

I.4. The prototype of cholesterol biosynthesis disorder-Smith Lemli-Opitz syndrome

In the last 30 years it has been revealed about several malformation syndromes (Smith-Lemli-Opitz syndrome, lathosterolosis, desmosterolosis) that they are caused by sterol synthesis error. The first one that has been documented is the Smith-Lemli-Opitz syndrome (SLOS), which was named after the medical doctors described it in 1964. The SLOS is an autosomal recessive, monogenetic malformation syndrome. The syndrome is caused by the deficiency of 7-dehydrocholesterol reductase (7-DHCR), which catalyzes the last step of the Kandutsch–Russell pathway.

Apart from decreasing the cholesterol level, the enzyme defect elevates the level of the precursor molecules of the cholesterol synthesis (most importantly 7-dehydrocholesterol (7DHC) and 8-dehydrocholesterol) both in the blood plasma and the cell membrane. As a result of the process, the composition of the lipid rafts and the membranes changes, different signaling pathways suffer from disorder, and the cholesterol as a universal precursor molecule (bile acids, neuroactive steroids, steroid hormones) cannot fulfill its task entirely either. However, it is still

an unanswered question, whether it is the lack of cholesterol or the toxic effect of the precursor molecules that are primarily responsible for the development of the symptoms.

The symptoms and occurrence of the disease are diverse: besides the frequent developmental disorders of the face and the head-neck region, almost all organ systems can be affected. The disease was originally described on the basis of the clinical phenotype as the simultaneous presence of facial dysmorphia, microcephaly, hypospadias and somatomental retardation by Smith, Lemli and Opitz. Besides the characteristics indicators of SLOS, the infant's abnormally slow weight-gain and feeding problems can draw the doctor's attention to the disease. Frequent consequences are the respiratory system's various bacterial and viral infections, which are rather caused by secondary factors (the anatomic deformation of the head-back region, the immobility of the patients, the hypotonia of the respiratory muscles) than the immunosuppression accompanying the disease. When clinical SLOS suspicion arises, it can be confirmed by the serum cholesterol decrease and the elevated 7DHC level. Further verification can be provided by molecular genetic testing. More than 130 different mutations of 7DHCR have been identified so far, but there is no tight genotype-phenotype correlation, thus the same mutation can lead to either serious or minor disorders. The patients' categorization in terms of the disease's seriousness is carried out with the help of the phenotype signs-based Bialer point system modified by Kelley and Hennekam. For the treatment of the disease only symptomatic therapies are available at present. Oral cholesterol substitution has been reported to improve behavioral problems and hearing functions, along with causing less photosensitivity and less infections. The antioxidant treatment and the statin therapy can be effective through the reduction of the sterol precursors' toxicity. Opinions differ about the effectivity of these treatments, since randomized, placebo controlled clinical studies have either yet to be done or they don't support the discrepancies found in non-standardized studies. There is no data about the incidence of the disease in Hungary yet, but the estimated values (1:10000-1:70000 or according to a recent research 1:39125) suggest that it is significantly underdiagnosed. The SLOS samples we investigated are from the previously characterized Hungarian patients.

I.5. Hypercholesterolemia and its immunological aspects

Hypercholesterolemia (HC), which develops during hyperlipidemia, is just a symptom. Three main reasons can be found in the background: overburdened metabolism due to the unhealthy diet and lifestyle; secondary metabolism disorders caused by diseases or medications; the primal, inherited disorders of lipid metabolism. In addition to cholesterol, the concentration of other lipids and lipoproteins also change, thus, along with etiology, the abnormalities can be

categorized according to the Fredrickson classification, which considers the lipoprotein concentration, or on the basis of the triglyceride and the cholesterol level.

The consequences of hypercholesterolemia can be, irrespective of etiology and classification, systemic arteriosclerosis, heart attack, stroke, or peripheral vasoconstriction. The effective treatment of hyperlipidemias is based on a suitable diet and a lifestyle change, which, in most cases, can be completed with the use of statin medications that selectively block the key enzyme of cholesterol biosynthesis called hydroxy-metil-glutanyl-CoA(HMGCoA).

We cannot overemphasize the general importance of hyperlipidemia, since in the developed countries more than 50 percent of the adult population have an abnormal lipid profile, and the accompanying diseases place a significant burden on the healthcare system. Although the recent years have shown a slight improvement, cardiovascular diseases are still the number one cause of death in Hungary. An intensive basic research have brought about in the past few years that unveiled the pathogenesis of atherosclerosis at molecular and cellular level. According to the most widely accepted hypothesis, arteriosclerosis is a chronical inflammatory reaction to the various damages of the endothelial cells, which are caused by mechanical stress, metabolic disorders, infections or smoking. The build-up of lipid (including cholesterol) molecules in the artery wall plays a significant role in maintaining the inflammation. The inflammatory factors produced by the damaged endothelium activate the inborn and adaptive immune system's cells, which, along with the smooth muscle cells, participate in the creation of atherosclerotic plaques saturated mainly with cholesterol. The plaque does not only block the vessels, but the detached fragments can also cause microemboli. On the top of that aneurysm frequently occurs in the weakened blood vessel wall, whose cracking can cause serious bleeding. It seems that T-cells play a prominent role in the inflammatory processes of arteriosclerosis. Both in human patients and model animals suffering from atherosclerosis the presence of activated T-cells in arteriosclerotic plaques was reported. A significant group of the T-cells was specific to oxidized lipid molecules. In hyperlipidemic animal models the depletion of $CD4^+$ lymphocytes reduced the progression of arteriosclerosis, while in immune deficient SCID mice the restoration of immunologic functions sped up the process. It is an important discovery, that the conditions leading to arteriosclerosis can also influence the normal functioning of the immune system, reducing its resistance towards various pathogens. The plasma hypercholesterinemia increases the cholesterol content of the lymphocytes' cell membrane. In the animal models the artificially generated hypercholesterinemia negatively influenced the physiological functions attributed to Kv ion channels. To our best knowledge, our work team has been the first to document the effect

of increased plasma hypercholesterinemia in the case of Kv1.3 channels in lymphocytes isolated from humane samples.

II. OBJECTIVES

In the recent years several studies have been published that characterize cholesterol as an important regulator of the ion channel function. In these studies the membrane cholesterol content was usually altered by *in vitro* methods (e.g. cyclodextrin-cholesterol complex), then the researchers investigated the impact of the altered environment on the ion channels. According to the previous results of our laboratory, the *in vitro* elevated cholesterol level modifies the gating of the Kv1.3 ion channels in lymphocytes isolated from healthy donors. As a result of the treatment, the activation and inactivation kinetics slowed down, and the equilibrium activation of the channel shifted towards the more positive membrane potentials. Similar changes have been described for calcium ion channels, too. One of the principal questions of our present work is whether the modifying role of sterols seen in *in vitro* experiments has any biological significance. Does any disease exist where the altered cholesterol content of the cell membrane influences the regulation of the ion channels? To answer this question, we studied the effect of two diseases (hypercholesteraemia and cholesterol synthesis disorder), which significantly but oppositely affect the cholesterol homeostasis, on the ion channels and function of T-cells isolated from peripheral blood.

Only little information is available about the function of the C-terminal of Kv1.3 channel. According to our hypothesis, the C-terminal of Kv1.3 can participate in the interaction of the channel with sterols, similarly to other ion channels (e.g. P2X7 and BK channels) Upon the investigation of the amino acid sequence of Kv1.3, we mapped several CARC cholesterol recognizing motifs in the C-terminal of Kv1.3. In our experiments we investigated the role of the C-terminal of Kv1.3 in the interactions with sterols.

In our work with focused on the following goals:

I. Analysis of the cholesterol content and the sterol profile of cells isolated from patients with hypercholesterinemia and Smith-Lemli-Opitz syndrome

II. Determine the gating parameters of Kv1.3 ion channels in lymphocytes isolated from patients with hypercholesterinemia or Smith-Lemli-Opitz syndrome

III. To explore the impact of the *in vitro* altered membrane cholesterol content on the T-lymphocyte functions regulated by ion channel (activation, proliferation)

IV. To model the membrane 7DHC content specific for SLOS in lymphocytes isolated from healthy donors

V. To study the biophysical features of Kv1.3 ion channels in the SLOS model system

VI. To compare the effects of cholesterol and by 7DHC on the Kv1.3 ion channel

VII. To examine the cholesterol and 7DHC sensitivity of the C-terminal deleted Kv1.3 ion channel

VIII. To reveal the cholesterol sensitivity of mutant channels with mutation in the CARC sequences located in the C-terminal of Kv1.3

III. MATERIALS AND METHODS

III.1. Reagents

All reagents were purchased from Sigma-Aldrich (Budapest, Hungary), unless stated otherwise.

III.2. Patients

III.2.1. Smith-Lemli-Opitz szindróma

Eight SLOS (age: 7.65±2.23 years) and eight age-matched control patients from Hungary (age: 7.66±1.83 years) were enrolled in the study.

III.2.2. Hypercholesterinaemia

The present study was performed on 7 healthy and 20 previously untreated type II/A and II/B hyperlipidemic patients. Hyperlipidemic patients were divided into two groups on the basis of total cholesterol level: slightly elevated hypercholesterolemic (5.2 mM < [total serum cholesterol] < 10 mM) and markedly elevated hypercholesterolemic groups ([total serum cholesterol] > 10 mM).

III.3. Cells

III.3.1. Mononuclear cells

Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. Cells were washed twice with Ca^{2+}- and Mg^{2+}-free Hanks' solution containing 25 mM HEPES (pH

7.4). Freshly isolated cells were used for CFSE-assay and the remaining mononuclear cells were frozen and stored at -80 ^{o}C until patch-clamp and CD154 expression experiments.

III.3.2. CHO (Chinese Hamster Ovary) cell line

CHO cells (ATCC, Germany) were cultured in DMEM medium, which also contained 10 % FBS, L-glutamine (2 mM), penicillin (100U/l), and streptomycin (100 mg/l). Cells were maintained at 37 °C in a humidified atmosphere of 5% of CO_2 and 95% air. Cells were passed every 2–3 days. CHO cells were transduced with retroviral particles containing mGFP-Kv1.3-WT and mGFP-Kv1.3ΔC constructs as described in (6).

III.4. 7DHC and CHOL loading of T cell membrane

Freshly isolated control (healthy) lymphocytes were washed twice with Hanks' solution and suspended in Hanks' solution (2×10^6 cells/ml) containing different concentrations of methyl-β-cyclodextrin/7-DHC (MβCD/7DHC**,** CycloLab Cyclodextrin Research and Development Laboratory Ltd. **,** Hungary) or methyl-β-cyclodextrin/cholesterol (MβCD/CHOL, CycloLab Cyclodextrin Research and Development Laboratory Ltd. , Hungary). In control experiments MβCD/7DHC, MβCD/C was not present in the solution. Cell suspensions were incubated for 1 h at 37 ^{o}C and washed three times with Hanks' solution.

III.5. Analysis of the cholesterol and 7-dehidrocholesterol levels of cells

III.5.1. Amplex Red Cholesterol Assay Kit

The cholesterol level of control and hypercholesterolemic cells was determined using Amplex Red Cholesterol Assay Kit according to the instructions of the manufacturer, and fluorescence was detected using Multimode Microplate Reader (BioTek, Winooski VT).

III.5.2. Analysis of the sterol composition of red blood cells using GC-MS

Gas chromatography-mass spectrometry (GC-MS) experiments were carried out by use of the standard protocols to define sterol composition of red blood cells isolated from SLOS. All of the GC-MS experiments were carried out by our collaborator, Mária Péter in Szeged. Identification was made based on CHOL and 7DHC standard solutions.

III.6. Electrophysiology

Whole-cell measurements were carried out using Axopatch-200A/B amplifiers connected to personal computers using Axon Digidata 1440 digitizers. For data acquisition and analysis the pClamp10 software package (Molecular Devices, Sunnyvale, CA) were used. $CD4^+$T

lymphocytes were selected for current recording by incubation with mouse anti-human CD4 antibodies (0.5 μg/10^6 cells, AMAC, Westbrook), followed by selective adhesion to petri dishes coated with goat anti-mouse IgG antibody (Biosource, Camarilo, CA), as described previously (5). Standard whole-cell patch-clamp techniques were used, as described previously (1). Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries (Clark Biomedical Instruments, UK) in four stages and fire-polished to give electrodes of 3–6 MΩ resistance in the bath. The standard bath solution (S-ECS) was (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.5 glucose, 10 HEPES (pH 7.35, 305 mOsm). The pipette solution was (in mM): 140 KF, 11 K_2EGTA, 1 $CaCl_2$, 2 $MgCl_2$, and10 HEPES (pH 7.20,~295 mOsm). To study activation kinetics of Kv1.3 T cells were depolarized to 50 mV for 15 or 30 ms from a holding potential –120mV. Kv1.3 current traces were fitted with a single exponential function rising to the maximum according to the Hodgkin-Huxley model ($I(t) = I_a \times (1-\exp(-t/\tau_a))^4 + C$, where I_a is the amplitude of the activating curve component;τ_a is the activation time constant of the current; C: current amplitude at –120 mV). The activation time constant for a particular cell was defined as the average of time constants obtained for at least 3 depolarizing pulses repeated at every 15 s in a sequence. We recorded at least four cells of a given patient or donor, and the average of the activation time constants was used to demonstrate the activation kinetics. To study the inactivation of Kv1.3 channels, two-second-long step pulses to +40 mV from a holding of -120 mV were applied to the cells. The fast activation of outward current is followed by a relatively slow decay of current corresponding to the C-type inactivation of this channel. The decaying part of the current traces was fitted with a single exponential function ($I(t) = I_0 \times \exp(-t/\tau_{in,i}) + C$, I_0: amplitude of current,$\tau_{in,i}$: inactivation time constant for different groups, C: steady-state value of whole-cell current at the end of the pulse) to attain the time constant characterizing the inactivation kinetics. The characteristic inactivation time constant for a given cell was determined as detailed for the activation kinetics. Membrane potential dependence of steady-state activation of the Kv1.3 current was determined as follows: cells were held at –120 mV and depolarized to gradually increasing test potentials. Peak whole-cell conductance (G(V)) at each test potential was calculated from the peak current (I_{peak}) at a test potential V and the K^+ reversal potential (E_K) using $G(V) = I_{peak}/(V_{test}-E_r)$. The G(V) values were normalized for the maximum conductance (G_{norm}) and plotted as a function of test potential along with the best-fit Boltzmann function ($G_{norm} = 1/(1+\exp[-(V-V_{½})/k]$, where G_{norm} is the normalized conductance, V is the test potential, $V_{½}$is the midpoint and k is the slope of the function). We calculated the steady-state

parameters at least four individual cells from each donor. The whole-cell membrane capacitance (C) setting on the amplifier required to cancel the transient charging currents evoked by periodic application of 5mV test pulses was considered as the membrane capacitance of the cells.

III.7. CD154 expression detection

Previously isolated control and SLOS mononuclear cells were stimulated with thapsigargin (1 μM) for 3 hours at 37 °C. For flow cytometry, cells were labeled with phycoerythrin conjugated mouse anti-CD3 antibody (PE-CD3) and Alexa-488 conjugated mouse anti-CD154 antibody (A488-CD154) and there isotype control for 20 minutes at room temperature according to the manufacturer's protocol (Biolegend; San Diego; CA). After the staining procedure, the fluorescence intensity of T cells was assessed by BD FacsScan flow cytometer (Beckton-Dickinson, NJ, USA). In all experiments, at least 10000 events were recorded and analyzed using FCS Express 4 software (De Novo Software, Glendale, USA).

III.8. CFSE staining and analysis

To study proliferation of lymphocytes, we applied the carboxyfluorescein succinimidyl ester (CFSE) dilution essay, originally described by Lyons et al (2-4). The final concentration of CFSE (CellTrace™ CFSE Cell Proliferation Kit, Life Technologies Co., Waltham, MA, USA) was 1μM that. After adding CFSE, we incubated the peripheral blood mononuclear cells (PBMC) for 15 minutes at room temperature, then for 20 minutes at 37°C. Lastly, the cells were washed once with PBS. We applied 500 ng/ml soluble anti-CD3 antibodies combined with 1 μg/ml soluble mouse anti-human CD28 for specific T cell stimulation in the mononuclear cell cultures. The flow cytometry analysis was performed on a BD FACScan™ cytometer. We measured the forward scatter (FSC), side scatter (SSC) and the fluorescence intensity in the green. Lymphocytes were selected from mixed cell populations of PBMC by their light scatter profile on FACS analysis. Cell proliferation was determined based on the decreasing CFSE intensity in the green channel. The indicator of proliferation was the division index (DI), calculated by the formula:

$$DI = \left(\sum_{k=1}^{n} A_k\right) / \left(\sum_{k=0}^{n} A_k\right)$$

where k points to the n^{th} generation of cells, and A_k is the cell number in the k^{th} division cycle according to.

III.9. Flanking primer mutagenesis

We applied modified version of flanking primer mutagenesis to generate point mutations in the wild type Kv1.3.

III.9.1. Polymerase chain reaction (PCR)

Phusion High-Fidelity DNA Polymerase enzyme was applied according to the manufacturer protocols (Thermo Fisher Scientific, Inc., Waltham). The following forward and reverse primers were used:

CARC4 binding site
forward:
5'GCGGTATCGGAGGCTGCGTGAGTCTCAGCTCAATGAGGA

reverse:
5'GCGTCGGAGGCTATGGCGATCGAAGAGGGGGGTATGAAC

CARC5 binding site
forward:
5' AGCATCGGTGGCTATCGCTGCGATGTTGACACAAGAGTTGGG
reverse:
5' GCAGCGATAGCCACCGATGCTTAAGCGGCCGCGAATTCTGCAGTC

III.9.2. Agarose gel electrophoresis

PCR products were separated in 1% agarose gels supplied with 0.5 μg/ml ethidium bromide. Gel tank was filled with 1xTAE buffer and the gel run at 100V for 30 min. DNA fragments were cut out under an UV transilluminator.

III.9.3. DNA extraction and purification

Traditional phenol extraction method was used to extract and purify DNA fragments.

III.9.4. Restriction digestion

Eco91I (GAATT↓C) and EcoRI (G↓GTAACC) restriction enzymes (Thermo Fisher Scientific, Inc., Waltham, USA) were applied according to the manufacturer's protocol.

III.9.5. Ligation, transformation, plasmid preparation

We used T4 DNA Ligase (Thermo Fisher Scientific, Inc., Waltham) for ligation with 1:5 vector:insert molar ratio. Circular plasmids were added to 200 µL E. coli (ET12567) component cell solution and incubated on ice for 20 minutes. Next, 42°C heat shock was applied for 1 min., finally cells were placed on ice for 2 min. Samples were suspended in 800µl SOC medium and cultured at 200 rpm and 37°C for 50 min in a shaking incubator. In the next step bacteria were spread onto LB agar plates containing 50 µg/ml kanamycin and incubated overnight, at 37°C. A single colony of transformed bacteria was inoculated into LB medium containing antibiotic and was grown at 37°C, 200 rpm overnight. PureYield Plasmid Miniprep System (Promega Corporation, USA) was used to purify plasmid DNA.

III.10. Data analysis

Prior to analysis whole-cell current traces were corrected for ohmic leak. Non-linear least squares fits were done using the Levenberg-Marquardt algorithm. Fits were evaluated visually as well as by the residuals and the sum of squared differences between the measured and calculated data points. Data are expressed as mean ±SEM. For pairwise comparisons Student's t-test or Mann–Whitney rank sum test (non-normally distributed populations) were applied. For multiple comparisons one-way or two-way ANOVA with post-hoc Holm-Sidak (HS) test were used. Statistical significance was taken as $p<0.05$.

IV. RESULTS

IV.1. Cholesterol content of lymphocytes in hypercholesterolemia

It has been previously demonstrated, that hypercholesterinemia increased the cholesterol content of T cells. To prove these results, we determined the cholesterol content of lymphocytes isolated from healthy donors and HC patients. To validate our technique, we introduced a positive control: normocholesterolaemic lymphocytes were loaded with cholesterol complexed to methyl-β-cyclodextrin (MβCD/C) at concentration of 1.5 mg/ml. The cholesterol concentration of samples from three different hypercholesterolaemic patients of group II is higher than the control value (control: 0.66 ± 0.009 µg/ml, n=3, group II: 0.91 ± 0.07 µg/ml, n=3, $p<0.001$). However, cholesterol loading using 1.5 mg/ml MβCD/C could

raise the cholesterol content of lymphocytes to a higher value than in cells obtained from group II of hypercholesterolemic patients (1.5 mg/ml MβCD/C: 1.53 ± 0.03 μg/ml, n=3, $p<0.001$).

IV.2. Expression of Kv1.3 channels in $CD4^+$ T lymphocytes in hypercholesterolemia

According to our previous study, *in vitro* cholesterol loading of lymphocytes decreases the Kv1.3 expression. Based on this, we hypothesized that the increased cholesterol content of hypercholesteremic T cells decrease the Kv1.3 expression. The expression level of Kv1.3 channels was measured by whole-cell current amplitudes (WCC) upon depolarization to + 50 mV for 15 ms from a holding potential of –120 mV. Current amplitudes were not different in control and hypercholesterolemic groups (WCC_c= 642.1 ± 44.3 pA, n=51, WCC_{GI}= 631.9 ± 48.2 pA, n=57, p=0.74; WCC_{GII}= 620.2 ± 37.4 pA, n=42, p=0.74). Hypercholesterolemia did not change the whole cell membrane capacitance of $CD4^+$ T lymphocytes ($C_{m,c}$= 1.68 ± 0.17 pF, n=31, $C_{m,GI}$= 1.67 ± 0.12 pF, n=46, p=0.96; $C_{m,GII}$= 1.54 ± 0.09 pF, n=42, p=0.48). The peak current normalized to membrane capacitance (current density, CuD) was calculated for each cell individually, which is the peak current at +50 mV divided by the membrane capacitance for each cell and these values were averaged for each group. Comparing the current densities we found no significant difference between normal and hypercholesterolemic $CD4^+$ T cells (CuD_c= 499.3 ± 65.4 pA/pF, n=39, p=0.49; CuD_{GI}= 450.7 ± 41.7 pA/pF, n=46, p=CuD_{GII}= 478.1 ± 45.6 pA/pF, n=42, p = 0.78). There was no significant difference between the CuD of Group I and II (p=0,708).

IV.3. Kinetic parameters of Kv1.3 gating in hypercholesterinemia

Lymphocytes were depolarized to + 50 mV for 15 ms from a holding potential of –120 mV. The activation kinetics of Kv1.3 current was fitted with a single exponential function rising to the maximum according to the Hodgkin-Huxley (HH) model ($I(t) = I_a \times (1-\exp(-t/\tau_a))^4 + C$) where I_a is the amplitude of the activating current component; τ_a is the activation time constant of the current; C: current amplitude at –120 mV). The activation time constant for a particular cell was defined as the average of time constants obtained for at least three consecutive depolarizing steps repeated at every 15 s. The activation kinetics of the whole-cell current was significant faster only in group II compared to control ($\tau_{a,c}$ = 0.811 ± 0.028 ms, n=51, $\tau_{a,GI}$ =0.731 ± 0.028 ms, n=47, $\tau_{a,GII}$ = 0.724 ± 0.024 ms, n=41, p=0.02). At 0 mV test potential, where the activation kinetics of the current is slower due to the inherent voltage-dependence of Kv1.3 gating we found significant acceleration of the activation

kinetics also in group I and II T cells compared to normocholesterolemic control. ($\tau_{a,c}$ = 2.28 ± 0.13 ms, n=50, $\tau_{a,GI}$ = 1.86 ± 0.09 ms, n=48, $\tau_{a,GII}$ = 1.79 ± 0.08 ms, n=40 p=0.002) . To study the inactivation kinetics of Kv1.3 current the decaying part of the current traces was fitted with a single exponential function ($I(t) = I_0 \times \exp(-t/\tau_{i,x}) + C$, where I_0 is amplitude of current, $\tau_{i,x,}$ is the inactivation time constant of the current in group x and C is steady-state value of whole-cell current at the end of the pulse) to obtain the time constant characterizing the inactivation kinetics. The inactivation time constant characteristic for given cell was defined as the average of time constants obtained for at least three consecutive depolarizing steps repeated at every 60 s. The inactivation was faster in hypercholesterolemic state but the statistical comparison did not show significant change versus control. ($\tau_{i,c}$ = 243.8 ± 8.0 ms, n=41; $\tau_{i,GI}$ = 229.3 ± 6.9 ms, n = 48; $\tau_{i,GII}$ = 234.9 ± 8.2 ms, n = 35, p = 0.43).

IV.4. Steady-state parameters of activation

In our previous study the *in vitro* cholesterol loading resulted in a rightward shift of the steady –state activation. Two parameters, the midpoint ($V_{1/2}$) and the slope factor (k) were used to characterize the steady-state activation. Our results showed that neither the midpoint of voltage dependence of steady-state activation ($V_{1/2,control}$ = –24.9 ± 0.87 mV, n=26, $V_{1/2}$,GI= –26.5 ±1.0 mV, n=38, p=0.25; $V_{1/2}$,GII = –26.1 ±0.9 mV, n=27, p=0.43) nor the slope factor of steady-state activation (sc=10.8 ± 0.35, n=26, sGI= 10.4 ± 0.22, n=38, p=0.32; sGII= 10.0 ± 0.26, n=27, p=0.07) was different among the control, group I and group II T cells.

IV.5. T cells in hypercholesterolemia show higher basal proliferation rate

T cell proliferation rate was determined using CFSE staining method (see Material and Methods) after antiCD3/antiCD28 stimulation. Our data shows that the antiCD3/antiCD28 stimulation of T lymphocytes was less effective in hypercholesterolemic state than that in the control group. The precursor frequency was 71.8 ± 10.2 % (n=10) in control group, 40.9 ± 5.1 % (n=7) in group I and 46.0 ± 5.0 % (n=11) in group II.

IV.6. Sterol composition of SLOS membrane

The Amplex-Red assay cannot differentiate between cholesterol and its oxidized forms. Therefore we applied GC-MS technique to characterize sterol profile of the red blood cells (RBC) from SLOS patients and age-matched control. Analysis of the sterol composition of the T lymphocyte cytoplasmic membrane was not possible due to the limited amount of the blood samples. On the other hand, erythrocytes do not have intracellular membrane systems,

hence, various CHOL precursors can only be incorporated into the plasma membrane. Therefore, the total sterol in RBCs should provide us with a good estimate of the sterol composition of the cytoplasmic membrane of other cells as well. In line with the previous results, we could identify elevated 7DHC and reduced CHOL levels in all of the SLOS samples, while 7DHC in the control samples was undetectable. 8DHC, which is a common isomer of 7DHC, was also identified in every SLOS sample. Results of the RBC sterol composition analysis are comparable to the comprehensive study by Russo et al (data not shown).

IV.7. Expression level of Kv1.3 channels in SLOS T cells is similar to that in healthy donors

In SLOS the reduced cholesterol levels associated with the increased concentrations of the precursor molecules of cholesterol synthesis (7DHC, 8DHC). The altered sterol composition of the cell membrane could modify the ion channel function. So far, there is no information about the effects of these SLOS derived precursors on Kv channel operation. Consequently we examined the expression and biophysical properties of Kv1.3 channels in SLOS patients. Current density (CuD: the peak current normalized to membrane capacitance, proportional to the number of channels per unit area) provides information about the impact of the channels on the membrane potential control of the cell, since a given number of channels regulate the membrane potential more readily in a smaller cell than in a larger one. T cells were held at –120 mV (holding potential) and depolarized to +50 mV for 15 ms in whole-cell configuration to determine whole cell peak currents (I_p), then I_p of an individual cell was divided by the membrane capacitance (C) of the particular cell to calculate current density. Whole-cell peak currents and whole-cell membrane capacitance were the same in control and SLOS groups ($I_{p,control}$ = 528.0 ± 69 pA, n = 56, 8 donors; $I_{p,SLOS}$ = 497.5 ± 31 pA, n = 51, 8 patients, p = 0.7 ; $C_{control}$ = 2.1 ± 0.1 pF, n = 56, 8 donors; C_{SLOS} =2.0 ± 0.2 pF; n= 51, 8 patients, p = 0.69).There was no statistical significant difference between the current densities of control and SLOS T cells ($CuD_{control}$ = 282.5 ± 42.9 pA/pF, n = 56, 8 donors; CuD_{SLOS} = 309.8 ± 39 pA/pF, n = 51, 8 patients, p = 0.65).

IV.8. Slowing of Kv1.3 current kinetics in SLOS T cells

The activation time constant (τ_a), and inactivation time constant (τ_i) were determined as we mentioned previously (see Materials and Methods). We found that the activation time constant was significantly greater for SLOS cells ($\tau_{a,control}$=0.59 ± 0.02ms, n = 56, 8 donors;

$\tau_{a,SLOS}$ = 0.75 ± 0.03ms, n = 51, 8 donor; p = 0.001), i.e. the kinetics of channel opening is slower in SLOS lymphocytes. In case of the inactivation we could not detect significant difference between SLOS and control cells increase ($\tau_{i,control}$ = 227.8 ± 14.2, n = 38, 8 donors; $\tau_{i,SLOS}$ = 253.7 ± 21.4, n = 45, 8 patients, p=0.329).

IV.9. SLOS modifies the membrane potential-dependence of steady-state activation

As the kinetics of the Kv1.3 gating was affected by the increased 7DHC level, we also investigated the equilibrium parameters of Kv1.3 activation. Test potential (V)-dependence of the normalized conductance (G_{norm}) elucidates the ratio of the open and closed channel pools at a given membrane potential, i.e. assesses the steady-state activation vs. membrane potential relationship (for details see Materials and methods). Comparison of the equilibrium activation parameters showed that the midpoint of the curve ($V_{1/2}$, the membrane potential at which 50% of the channels is open at steady-state) is significantly more positive for SLOS T cells ($V_{½,control}$= –26.9 ± 0.9 mV, n = 41, 8 donors; $V_{½,SLOS}$ = –22.0 ± 1.9 mV, n = 35, 8 patients; p = 0.03), without a statistically significant change in the slope factor ($k_{control}$ = 11.7 ± 0.5 mV, n =41, 8 donors; k_{SLOS} =10.6 ± 0.5, 8 patients; p=0.14).

IV.10. Activation and proliferation of SLOS T cells is suppressed

We aimed to study the effects of the altered CHOL/7DHC ratio on the Kv1.3-dependent biological functions of T cells such as activation and proliferation. To assess the effect of SLOS on T cell activation, we measured the expression of CD154 of lymphocytes. CD154 is a marker of activation on T lymphocytes stimulated through calcium-triggered NFAT pathways. We assumed that the modified ion channel function in SLOS influences the Kv1.3-dependent calcium signaling during T cell activation, which could change the expression of CD154. Our results indicate that the ratio of $CD154^+$ T cells decreased significantly in SLOS compared to the age-matched healthy controls (fraction of $CD154^+CD3^+$cells in SLOS: 8.6 ± 3.2%, 5 patients; control: 21.1 ±2.8%, 4 donors, p<0.001). We also tested the proliferation capacity of T cells using the CFSE staining technique, which is a suitable method to observe the proliferation characteristics of lymphocytes with flow cytometry. We found that the five-day antiCD3/antiCD28 stimulation (see details in Materials and Methods) of T lymphocytes was less effective in SLOS than in the control group. The division indices/indexes of T lymphocytes were 29.4 ± 3.5% (n = 6) and 61.1 ± 7.1 % (n = 5) for SLOS and the control group, respectively (p = 0.002).

IV.11. Modeling the SLOS sterol environment in T cells

In order to verify if 7DHC incorporation into the T cell membrane affects Kv1.3 function, we created an SLOS-like sterol environment by modification of the 7DHC content of healthy T cells with MβCD/7DHC complex *in vitro*. To validate the incorporation of 7DHC into the plasma membrane, untreated and MβCD/7DHC treated PBMCs were subjected to GC-MS analysis. We applied 140μM MβCD/CHOL as a positive control for sterol-loading of the cells. We found that the amount of 7DHC in the cell membrane increases gradually with the higher applied concentration of MβCD/7DHC (7DHC level normalized to protein content was 26.1±1.00 μg/mg for 65μM MβCD/7DHC and 66.8±11 μg/mg for 195μM MβCD/7DHC). Parallel to the increase in MβCD/7DHC, the CHOL content showed decreasing tendency, which can be due to the CHOL removal by the empty MβCD, but the change was not significant as compared to the untreated group (CHOL level normalized to protein amount was 18.3 ± 3.71 μg/mg for control, 16.2±0.2 μg/mg for 65μM MβCD/7DHC; 13.3±2 μg/mg for 195μM MβCD/7DHC). Thus, MβCD/7DHC-treated T lymphocytes reproduce the sterol environment found in SLOS, consequently, these T-cells can serve as a model for SLOS conditions.

IV.12. Biophysical properties of Kv1.3 in 7DHC-loaded T cells resemble those observed in SLOS

Next we characterized the kinetic and equilibrium parameters of Kv1.3 gating in the 7DHC-loaded T cells. First, the activation kinetics of Kv1.3 channels was measured for 7DHC-loaded cells and we found that activation kinetics became slower as the 7DHC level increased in the membrane (τ_a = 0.59 ± 0.02 ms for control (n=56); 1.76 ± 0.18 ms for 32.5 μM MβCD/7DHC (n=7), $p < 0.001$; 2.04 ± 0.15 ms for 65 μM MβCD/7DHC (n=11), $p<0.001$; 2.70 ± 0.26 ms for 195 μM MβCD/7DHC (n=11), $p<0.001$). For the inactivation kinetics, we found that the time constant increased compared to untreated cells (τ_i = 227.8 ± 14.2 ms for control (n = 38); 318.57 ± 22 ms for 32.5 μM MβCD/7DHC (n = 5), p = 0.064, 339 ± 57 ms for 65μM MβCD/7DHC (n = 6), p = 0.02; 362 ± 26.4 ms for 195μM MβCD/7DHC (n = 5), $p<0.01$). Finally, the voltage-dependence of steady-state activation was evaluated for T cells treated with different amounts of MβCD/7DHC: just as described for SLOS cells, the midpoint of activation, i.e. the half-maximal voltage was shifted to the positive voltages ($V_{½}$ was −26.9 ± 0.9 mV for control (n = 41); −19.2 ± 1.3 mV for 32.5 μM MβCD/7DHC (n = 6), $p<0.01$; −13.4 ± 2.6 mV (n = 9) for 65 μM MβCD/7DHC, $p<0.001$; −10.1 ± 1.9 mV for

195 μM MβCD/7DHC (n = 9), p<0.001) unlike the slope factor, which was the same for all treatments (k = 11.7 ± 0.5 mV for control (n = 41); 11.07 ± 0.11 mV for 32.5 μM MβCD/7DHC (n = 6), p = 0.53; 12.48 ± 0.56 mV for 65 μM MβCD/7DHC (n = 9), p = 0.34; 13.58 ± 0.80 mV for 195 μM MβCD/7DHC (n = 9), p = 0.03). Current density, whole-cell peak current and membrane capacitance (see above) were also determined for 32.5, 65, and 195 μM MβCD/7DHC treatments. Although membrane capacitance did not change, the whole-cell peak currents decreased significantly upon the 7DHC loading (C was: 2.12 ± 0.12 pF for control (n = 51); 2.20 ± 0.22 pF for 32.5μM MβCD/7DHC (n = 7), p = 0.80; 2.02 ± 0.29 pF for 65 μM MβCD/7DHC (n = 11), p = 0.81; 2.26 ± 0.21 pF for 195 μM MβCD/7DHC (n = 11), p = 0.65; $I_p$was: 527.7 ± 68.7 pA for control (n = 51); 296.5 ± 52.5 pA for 32.5 μM MβCD/7DHC (n = 7), p = 0.002; 300.0 ± 37.4 pA 65 μM MβCD/7DHC (n = 11), p = 0.003; 174.8 ± 9.1 pA for 195 μM MβCD/7DHC (n = 11), p<0.001). Consequently, the MβCD/7DHC loading significantly decreased the current density of Kv1.3 of T cells unlike for those from the SLOS patients (CuD was 282.5 ± 42.9 pA/pF for control (n = 51); 153.0 ± 44.8 pA/pF for 32.5 μM (n = 7) p = 0.005; 176.9 ± 26.5 pA/pF for 65 μM (n = 11), p = 0.002; and 81.9 ± 8.9 pA/pF for 195 μM, p<0.001, (n = 11).

IV.13. Comparison of CHOL and 7DHC loading

In our previous study we demonstrated that *in vitro* loading of CHOL slowed the kinetics and modified the steady-state parameters of Kv1.3 gating. Similar changes in the biophysical parameters of Kv1.3 gating were found in the current study in SLOS and SLO model system. These results raised the question whether the effect of 7DHC and CHOL loading are quantitatively similar or different. Therefore, we treated the T cells with the same concentration of CHOL and 7DHC and measured the biophysical properties of Kv1.3. We found that Kv1.3 is more sensitive to 7DHC than CHOL loading at 32.5μM: 7DHC significantly increased the activation time constant and shifted $V_{1/2}$ towards positive voltages, whereas CHOL did not (τ_a = 0.59 ± 0.02 ms for control (n = 56); 0.75±0.15 ms for 32.5 μM MβCD/CHOL (n = 5), p = 0.40; 1.76±0.18 ms for 32.5 μM MβCD/7DHC (n = 5), p<0.001;$V_{1/2}$ = −26.9 ± 0.9 mV for control (n = 41); −24.4 ±3.1 mV for 32.5 μM MβCD/CHOL (n = 5), p = 0.318; and −19.2 ± 1.3 mV for 32.5 μM MβCD/7DHC (n = 6), p = 0.004). At higher concentrations of MβCD/7DHC and MβCD/CHOL both compounds changed the kinetic and steady state parameters of Kv1.3 gating but 195 μM MβCD/7DHC was more effective in changing $V_{1/2}$ than equimolar MβCD/CHOL ($V_{1/2}$ = −17.4 ±0.58 mV

for 65 μM MβCD/CHOL(n = 5), p = 0.29; and −13.4±2.6 mV for 65 μM MβCD/7DHC (n=5), p = 0.024; −16.2 ± 2.72 mV for 195μM MβCD/CHOL (n = 5), p = 0.038; and −10.1 ± 1.9 mV for 195 μM MβCD/7DHC (n=5), p<0.001; τ_a = 0.99 ± 0.09 ms for 65 μM MβCD/CHOL (n=5), p = 0.059; and 2.04 ± 0.15 ms for 65 μM MβCD/7DHC (n = 5), p<0.001;1.05±0.09 ms for 195μM MβCD/CHOL(n = 5), p = 0.02; and 2.62 ± 0.22 ms for 195 μM MβCD/7DHC(n = 5), p<0.001).

IV.14. Removal of the cytoplasmic C-terminal of Kv1.3: loss of sensitivity to sterol loading

We tested the effect of CHOL and 7DHC loading on a C-terminal-deleted Kv1.3 channel (mGFP-Kv1.3ΔC), which lacks the last 84 amino acid residues at its C-terminal and consequently lacks the two CARC sequences recently identified on other ion channels as specific CHOL receptor sites being responsible for the effect of sterols on ion channels. Previously we showed that biophysical characteristics of the Kv1.3-WT and Kv1.3ΔC are identical, removal of the C-terminal did not change significantly gating properties of Kv1.3. After that Kv1.3-WT and Kv1.3ΔC expressing CHO cells were loaded using 420μM CHOL and 7DHC, and ionic currents were measured in outside-out patch configuration. As expected 420μM CHOL or 7DHC shifted the midpoint of the voltage-dependence of steady-state activation and increased the activation time constant in case of the mGFP-Kv1.3-WT CHO cells ($V_{1/2,WT}$: −28.87±1.52 (n = 5) for control and −17.45±1.90 mV for 420 μM CHOL (n = 7), p<0.001; −19.25±4.72 mV (n = 5), p = 0.028 for 420 μM 7DHC; $\tau_{a,WT}$: 0.45±0.014 ms for control (n = 5), 0.76±0.022 ms for 420 μM MβCD/CHOL (n = 7) p = 0.002; 0.69±0.059 ms for 420μM MβCD/7DHC (n = 10) p = 0.008). On the contrary, mGFP-Kv1.3ΔC channels were insensitive to both CHOL and 7DHC loading ($V_{1/2,\Delta C}$:−26.73±2.72 mV for control (n = 6); $V_{1/2,\Delta C}$:−26.30±2.72 mV for 420 μM MβCD/CHOL (n = 8), p>0.05; $V_{1/2,\Delta C}$: −23.05±1.08 mV for 420 μM MβCD/7DHC (n = 6), p>0.05); and $\tau_{a,\Delta C}$: 0.59±0.043 ms for control (n = 6), 0.55±0.022 ms for 420μM MβCD/CHOL (n = 7), p>0.05; 0.65±0.036 ms for 420μM MβCD/7DHC (n = 10), p>0.05). Intracellular C-terminus is less sensitive to the changes of the physical properties of the cell membrane relative to transmembrane regions. Based on this, we assumed that cholesterol could affect the gating of Kv1.3 not only via regulating physical properties of the cell membrane but also creating specific lipid-protein interactions. Scanning through the C-terminus of Kv1.3 we could identify two potential cholesterol recognition sites (CARC1 and CARC2). To study the significance of these motifs we changed the key amino

acid residues to alanine. (In case of the $Kv1.3_{CARC1}$ 474lysine, 479tyrosine and 479valine while in $mKv1.3_{CARC2}$ 517lysine, 518lysine, 520phenylalanine, 523valine were mutated to alanine.) As we expected based on the biophysical characteristics of the Kv1.3ΔC channel, $Kv1.3_{CARC1}$ and $mKv1.3_{CARC2}$ had similar gating properties as compared to the wild-type Kv1.3. Then we loaded the transfected CHO cells with 420μM CHOL or 7DHC and determined the biophysical properties of $mKv1.3_{CARC1}$ and $mKv1.3_{CARC2}$. Point mutations have changed the sterol sensitivity of the mutated channels. In the case of $mKv1.3_{CARC1}$ the steady-state activation shifted rightward while the activation kinetics did not alter upon cholesterol loading. The same amount of 7DHC loading significantly increased the activation time constant and the half-maximal voltage of the steady state activation ($V_{1/2,\ control}$: −26.37±1.6 (n = 8) for control and −17.09±2.27 mV for 420 μM CHOL (n = 8), p=0.005; −18.89±1.94 mV (n = 3), p = 0.03 for 420 μM 7DHC; $\tau_{a,control}$: 0.57±0.03 ms for control (n = 14), 0.63±0.04 ms for 420 μM MβCD/CHOL (n = 14) p = 0.248; 0.98±0.06 ms for 420μM MβCD/7DHC (n = 3) p = 0.001). The cholesterol loading slowed the activation kinetics of $mKv1.3_{CARC2}$ but it did not alter the steady-state activation. The 7DHC treatment caused similar changes that we found in case of the wild type channel. ($V_{1/2,\ control}$: −26.45±3.3 (n = 5) for control and −24.26±2.27 mV for 420 μM CHOL (n = 9), p=0.59; −16.23±1.61 mV (n = 5), p = 0.024 for 420 μM 7DHC; $\tau_{a,control}$: 0.44±0.01 ms for control (n = 11), 0.63±0.04 ms for 420 μM MβCD/CHOL (n = 14) p = 0.001; 1.59±0.31 ms for 420μM MβCD/7DHC (n = 5) p < 0.001).

V. SUMMARY

Cholesterol is an essential component of the cell membrane and involved in the syntheses of many bioactive molecules. Hypercholesterinemia, which is characterized by the abnormally elevated serum cholesterol level, is a frequent dyslipidemia. Inborn errors of cholesterol synthesis (e.g. Smith-Lemli-Opitz syndrome, SLOS) are rare, genetic disorders with pathognomonic low cholesterol and increased cholesterol precursor serum levels. *In vitro* manipulation of the membrane cholesterol of T cells modifies the gating parameters of the essential voltage-gated Kv1.3 channel, however a comprehensive study that confirms the pathophysiological significance of these results is missing. Therefore we isolated lymphocytes from patient with SLOS and hypercholesterinemia to study the consequence of *in vivo* altered membrane cholesterol composition on Kv1.3 channel and T lymphocyte function. To expand our knowledge about the interaction of sterols with Kv1.3 we searched for the existence of cholesterol recognition sites in an *in vitro* modified sterol environment. Our results indicated that hypercholesterinemia just slightly altered the Kv1.3 function unlike in our previous study where *in vitro* cholesterol loading had remarkable effects. The reason of this could be that the cholesterol content of the cells in hypercholesterolemia is not as high as in the *in vitro* model systems. This is supported by the data obtained in a patient with extreme high cholesterol level: changes in Kv1.3 biophysical parameters were comparable to those described for the *in vitro* loaded T cells. The proliferation capacity of the T cells decreased regardless of the lack in the Kv1.3 function.

In SLOS the kinetics of Kv1.3 gating slowed down and the steady-state activation shifted toward positive potentials. Identical changes in Kv1.3 operation were observed when control/healthy T cell membrane was loaded with 7DHC. Functional assays exhibited impaired activation and proliferation rate of T cells probably partially due to the modified Kv1.3 operation. Removal of the putative sterol binding sites on Kv1.3 resulted in a phenotype that was not influenced by the elevation in membrane sterol level. We suppose that in hypercholesterinemia only the extreme high serum cholesterol levels influence biological functions of Kv1.3 significantly. We also concluded that the altered membrane sterol composition in SLOS hindered the operation of Kv1.3 as well as the ion channel-controlled T cell functions. We propose that the ion channel-sterol interaction described in our study reveals a molecular mechanism that may contribute to the pathophysiological conditions in SLOS, and may lead to the most prominent neurological and cardiovascular symptoms via influencing the physiological function of ion channels.

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List of publications related to the dissertation

1. **Balajthy, A.**, Somodi, S., Pethő, Z., Péter, M., Varga, Z., Szabó, G., Paragh, G., Vígh, L., Panyi, G., Hajdu, P.: 7DHC-induced changes of Kv1.3 operation contributes to modified T cell function in Smith-Lemli-Opitz syndrome.
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IF: 3.654 (2015)

2. Somodi, S., **Balajthy, A.**, Szilágyi, O., Pethő, Z., Harangi, M., Paragh, G., Panyi, G., Hajdu, P.: Analysis of the K+ current in human CD4+ T lymphocytes in hypercholesterolemic state.
Cell. Immunol. 281 (1), 20-26, 2013.
DOI: http://dx.doi.org/10.1016/j.cellimm.2013.01.004
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List of other publications

3. Olamendi-Portugal, T., Bartók, Á., Zamudio, F. Z., **Balajthy, A.**, Becerril, B., Panyi, G., Possani, L. D.: Isolation, chemical and functional characterization of several new K+-channel blocking peptides from the venom of the scorpion Centruroides tecomanus.
Toxicon. 115, 1-12, 2016.
DOI: http://dx.doi.org/10.1016/j.toxicon.2016.02.017
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4. Pethő, Z., **Balajthy, A.**, Bartók, Á., Bene, K., Somodi, S., Szilágyi, O., Rajnavölgyi, É., Panyi, G., Varga, Z.: The anti-proliferative effect of cation channel blockers in T lymphocytes depends on the strength of mitogenic stimulation.
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