



Analysis of the K⁺ current in human CD4⁺ T lymphocytes in hypercholesterolemic state

Sándor Somodi^a, András Balajthy^b, Orsolya Szilágyi^b, Zoltán Pethő^b, Mariann Harangi^a, György Paragh^a, György Panyi^b, Péter Hajdu^{b,*}

^a 1st Department of Internal Medicine, University of Debrecen, Medical and Health Science Center, Nagyerdei krt. 98, H-4012, Hungary

^b Department of Biophysics and Cell Biology, University of Debrecen, Medical and Health Science Center, Nagyerdei krt. 98, H-4012, Hungary

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ABSTRACT

Atherosclerosis involves immune mechanisms: T lymphocytes are found in atherosclerotic plaques, suggesting their activation during atherogenesis. The predominant voltage-gated potassium channel of T cells, Kv1.3 is a key regulator of the Ca²⁺-dependent activation pathway. In the present experiments we studied the proliferation capacity and functional changes of Kv1.3 channels in T cells from healthy and hypercholesterolemic patients.

By means of CFSE-assay (carboxyfluorescein succinimidyl ester) we showed that spontaneous activation rate of lymphocytes in hypercholesterolemia was elevated and the antiCD3/antiCD28 co-stimulation was less effective as compared to the healthy group. Using whole-cell patch-clamping we obtained that the activation and deactivation kinetics of Kv1.3 channels were faster in hypercholesterolemic state but no change in other parameters of Kv1.3 were found (inactivation kinetics, steady-state activation, expression level). We suppose that incorporation of oxLDL species via its raft-rupturing effect can modify proliferative rate of T cells as well as the gating of Kv1.3 channels.

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1. Introduction

Hypercholesterolemia (elevated total cholesterol and LDL-cholesterol level) is the most important risk factor for atherosclerosis which leads to cardiovascular diseases (CVD). Cardiovascular diseases are characterized by a formation of atherosclerotic plaques consisting of necrotic cores, calcified regions, accumulated modified lipids, inflamed smooth muscle cells, endothelial cells, leukocytes, and foam cells [1]. Atherosclerosis is a complex disease, and many components of the vascular, metabolic, and immune systems are involved in this process. The presence of leukocytes within atherosclerotic arteries was reported in the early 1980s [2]. Initially, investigators thought that only macrophages are predominantly present within atherosclerotic vessels. However, several studies reported the presence of most known leukocytes in both mouse and human aortas [3,4]. Oxidized lipoprotein- and heat shock protein (HSP)-specific T cells are found in atherosclerotic plaques, suggesting local activation and clonal expansion of

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; CD, cluster of differentiation; CVD, cardiovascular disease; Hsp, heat shock protein; HPB, human peripheral blood; TCR, T cell receptor; T_H1, T_H2, helper T lymphocytes type 1 and 2; MβCD, methyl beta cyclodextrin; MβCD/C, methyl-beta-cyclodextrin/cholesterol complex.

* Corresponding author. Fax: +36 52 412623.

E-mail address: hajdup@dote.hu (P. Hajdu).

T lymphocytes during atherogenesis [5,6]. Several studies suggest that the T_H1 response is proatherogenic and affects atherosclerosis not only through the production of proinflammatory cytokines, but also through the regulation of B cell function and antibody production [7,8].

Furthermore, cholesterol influences numerous signaling pathways, including T and B cell activation [9,10], cytokine, and chemokine stimulation [11,12], growth factor signaling [13], cell migration [14], neuronal signaling [15], and apoptotic pathways [16]. Cholesterol is one of the main components of lipid rafts, which are glycosphingolipid-enriched detergent resistant membrane microdomains. Cholesterol is thought to serve as a space between the hydrocarbon chains of the sphingolipids and to function as a dynamic glue that keeps the raft assembly together. Removal of raft cholesterol leads to dissociation of most proteins from rafts and renders them non-functional. Vayá and co-workers reported that patients with primary hypercholesterolaemia show increased erythrocyte membrane cholesterol and phospholipid content [17]. The cholesterol content of membrane of T lymphocytes influences T cell proliferation. The increased membrane cholesterol content with aging inhibit T cell signaling through lipid rafts by impairing the recruitment of signaling proteins [18]. *In vitro* cholesterol loading using methyl-beta-cyclodextrin/cholesterol (MβCD/C) complex impaired T cell activation and chemokine stimulation [19].

The voltage-gated K⁺ channel Kv1.3 plays a key role in the activation of human T cells, as demonstrated by the inhibition of T cell mediated immune responses by compounds blocking this channel [20,21]. Another potassium channel, gated by the intracellular Ca²⁺ concentration was also identified in T cells, and was named IKCa1 for intermediate conductance Ca²⁺-activated K⁺ channel or KCa3.1 [22]. Following antigenic stimulation a sustained Ca²⁺ signal is required in T cells, during which the membrane potential is stabilized by these two K⁺ channel types [23]. Changes in the level of membrane cholesterol were shown to regulate multiple types of K⁺ channels including the Kv1.3 channels of lymphocytes. Bock et al. showed that Kv1.3 channels partition almost exclusively to cholesterol-rich membrane domains in a T-lymphocytes cell line and that cholesterol depletion significantly decreases Kv1.3 activity [24]. In our previous study we observed an opposite effect in human peripheral blood (HPB) T lymphocytes upon loading the membrane with cholesterol using the methyl-beta-cyclodextrin/cholesterol (M β CD/C) complex. Specifically, elevation of membrane cholesterol in HPB T lymphocytes resulted in a significant decrease in Kv1.3 current density, slowing of both activation and inactivation kinetics and a right-shift in the voltage-dependence of steady-state activation [25]. These changes are consistent with a decrease in the open probability of the channels upon cholesterol loading. Our group also demonstrated the clustering of Kv1.3 channels with a T cell antigen receptor complex [26] and localization of Kv1.3 to the immunological synapse where it also colocalizes with a lipid raft marker GM1 [27]. Kv1.3 also partitions into lipid rafts when expressed heterologously in HEK cells but its membrane distribution is uniform rather than clustered [28], which may be consistent with partitioning into small but highly abundant lipid rafts.

In our present study we aimed to determine the effect of hypercholesterolemia on the T cell proliferation and on the expression and gating of Kv1.3 channels. Our research was motivated by the fact that although the effect of cholesterol on lymphocyte proliferation and Kv1.3 gating is well characterized in model systems where cholesterol loading of the cells was accomplished using M β CD/C, it is not known whether these changes are present in lymphocytes of patients with hypercholesterolemia.

2. Materials and methods

2.1. Patients

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) (see Results for details). Patients with hepatic, endocrine or renal disorders (serum creatinine level > 130 μ mol/L), type 2 diabetes mellitus, alcoholism, drug dependence, gallstones, malignancy, pregnancy or lactation, or patients on anticoagulant or lipid-lowering therapy were excluded from the study. Patients were informed and all signed the informed consent form.

2.2. Cells

Ten milliliter heparinized human peripheral venous blood was obtained from each patient. Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. Collected cells were washed twice with Ca²⁺- and Mg²⁺-free Hanks' solution containing 25 mM HEPES (pH 7.4). After separation mononuclear cells were frozen and stored at -78 °C until measurements.

2.3. Electrophysiology

Whole-cell measurements were carried out using Axopatch-200 and Axopatch-200A amplifiers connected to personal computers using Axon Instruments Digidata 1440 or 1322A data acquisition boards. For data acquisition and analysis the pClamp9 and the pClamp10 software package (Axon Instruments Inc., Foster City, CA) were used. CD4 positive T lymphocytes were selected for current recording by incubation with mouse anti-human CD4 antibodies (0.5 μ g/10⁶ cells, AMAC, Westbrook), followed by selective adhesion to petri dishes coated with goat anti-mouse IgG antibody (Biosource, Camarillo, CA), as previously described by Matteson and Deutsch and Deutsch and Chen [21,29]. Dishes were washed gently five times with 1 ml of normal extracellular bath medium (see below) before the patch-clamp experiments. Standard whole-cell patch-clamp techniques were used, as described previously [30]. Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries (Clark Biomedical Instruments, UK) in five stages and fire-polished to give electrodes of 2–3 M Ω resistance in the bath. The standard bath solution (S-ECS) was (in mM): 145 NaCl, 5 KCl, 1 MgCl₂, 2.5 CaCl₂, 5.5 glucose, 10 HEPES (pH 7.35, 305 mOsm). The pipette solution was (in mM): 140 KF, 11 K₂EGTA, 1 CaCl₂, 2 MgCl₂, and 10 HEPES (pH 7.20, ~295 mOsm). Series resistance compensation up to 85% was used to minimize voltage errors and achieve good voltage clamp conditions (V_{err} < 5 mV).

2.4. CFSE staining and analysis

Proliferation activity of T cells was measured in FACScan flow cytometer using CFSE (carboxyfluorescein succinimidyl ester) dilution assay. Final concentration of CFSE was 1 μ M. The cells were cultured in 96 well plates (0.5 $\times$ 10⁶ cell/well) in RPMI 1640 + 10% FCS and stimulated with antibodies affecting the TCR-CD3 complex (antiCD3, 1 μ g/ml, OKT3, ATCC) and antiCD28 (1 μ g/ml, BD Biosciences) used in combination). Five days after the stimulus we measured the proportion of proliferating cells based on the change in fluorescence intensity in duplicates or triplicates [31]. Basal activity of T cells was measured on the basis of side-scatter and forward-scatter values. Activated lymphocytes are larger and more granulated, than resting cells. To measure the effectivity of antiCD3/antiCD28 stimulation the precursor frequency was calculated, which means the frequency of proliferating cells in the original population (before culture) that had proliferated. Precursor frequency was calculated according to method described by Gilvan et al. [32].

2.5. Cell cholesterol content measurement

The cholesterol level of control and hypercholesterolemic cells was determined using Amplex Red Cholesterol Assay Kit. The cells were lysed (composition of lysis solution: 50 mM Tris (pH 8.0), 2 mM CaCl₂, 80 mM NaCl, 1% Triton X-100), then for the fluorometric quantification, 25 μ l of cell extracts or cholesterol standards were incubated in 96-well plates with the reagents according to the instructions of the manufacturer, and fluorescence was detected using Multimode Microplate Reader (BioTek, Winooski VT). Using different concentration of cholesterol standard calibration curve was fitted to data points. Cholesterol concentration of samples was calculated using calibration curve.

2.6. Data analysis

Prior to analysis whole-cell current traces were corrected for ohmic leak and digitally filtered (three point boxcar smoothing). Non-linear least squares fits were done using the Marquardt–Levenberg algorithm. Fits were evaluated visually as well as by

the residuals and the sum of squared differences between the measured and calculated data points.

Statistical comparisons were made using Analysis of Variance (ANOVA) or Student's *t*-test at $p = 0.05$. For all experiments, the standard error of the mean (SEM) is reported for at least $n = 3$.

3. Results

3.1. Classification of patients into groups

Patients and healthy volunteers involved in this study were categorized into three groups on the basis of the total serum cholesterol level. The control group contains normocholesterolemic patients (total cholesterol level lower than 5.2 mmol/l ($n = 7$, age: 37.7 ± 2.22 years, BMI: 25.5 ± 1.14 kg/m²). Patients of group I have total cholesterol level between 5.2 and 10 mmol/l ($n = 11$, age: 44.0 ± 4.8 year, BMI: 26.0 ± 0.9 kg/m²). Patients with total cholesterol more than 10 mmol/l were categorized into group II ($n = 9$, age: 54.9 ± 3.1 year, BMI: 27.5 ± 1.6 kg/m²). In group I the total cholesterol level was 7.2 ± 0.35 mmol/l, LDL-cholesterol level was 4.6 ± 0.3 mmol/l, while triglyceride level 3.9 ± 1.8 mmol/l ($n = 11$). In group II the total cholesterol, LDL-cholesterol and triglyceride levels were 11.9 ± 0.7 , 7.8 ± 0.7 and 8.8 ± 2.5 mmol/l, respectively ($n = 9$).

3.2. T cells in hypercholesterolemia show higher basal proliferation rate

T cell proliferation rate was determined using CFSE staining method (see Material and Methods). T lymphocytes of group I showed higher activity without any stimulation than those belong to the control group: the ratio of activated T cells was $7.2 \pm 0.34\%$, ($n = 7$ individuals) in control group, $12.2 \pm 2.08\%$ in Group I, while $11.0 \pm 1.6\%$ ($n = 7$ patients) in Group II (Fig. 1A, $p = 0.03$). After antiCD3/antiCD28 stimulation the proliferation rate of lymphocytes showed high donor variability. The peak precursor frequency on a given day was between 20.6% and 50.9%. For better comparison of results we normalized the measured precursor frequencies to the highest precursor frequency measured on the given day. 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 $71.8 \pm 10.2\%$ ($n = 10$) in control group, $40.9 \pm 5.1\%$ ($n = 7$) in group I and $46.0 \pm 5.0\%$ ($n = 11$) in group II ($p = 0.015$, Fig. 1B).

3.3. Expression of Kv1.3 channels in CD4⁺ T lymphocytes in hypercholesterolemia

Kv1.3 channel is the predominant voltage-gated potassium channel of T lymphocytes, which controls the proliferation activity

of them. To investigate the background of altered proliferation activity of T cells in hypercholesterolemia we examined the expression and biophysical properties of Kv1.3 channels. 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, $n = 57$, WCC_{GII} = 620.2 ± 37.4 pA, $n = 42$, $p = 0.82$). 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, $n = 46$, C_{m,GII} = 1.54 ± 0.09 pF, $n = 42$, $p = 0.882$). 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, $n = 39$, CuD_{GI} = 450.7 ± 41.7 pA/pF, $n = 46$, CuD_{GII} = 478.1 ± 45.6 pA/pF, $n = 42$, $p = 0.844$).

3.4. Kinetic parameters of Kv1.3 activation, inactivation and deactivation in normal and high-cholesterol patients

Fig. 2A shows normalized current traces recorded in T lymphocytes from a normocholesterolemic and a group II hypercholesterolemic individual upon depolarization to +50 mV for 15 ms from a holding potential of −120 mV. Pulses were delivered to the cells in every 15 s to avoid cumulative inactivation. Normalization of current recordings to the peak value was introduced for easier comparison of the kinetic parameters of the currents. 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 [33]. 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 (Fig. 2B; $\tau_{a,c} = 0.811 \pm 0.028$ ms, $n = 51$, $\tau_{a,GI} = 0.731 \pm 0.028$ ms, $n = 47$, $\tau_{a,GII} = 0.724 \pm 0.024$ ms, $n = 41$, $p = 0.04$). At 0 mV test potential, where the activation kinetics of the current is slower due to the inherent voltage-dependence of Kv1.3 gating [21] we found significant acceleration of the activation kinetics also in group I and II T cells compared to normocholesterolemic control. ($\tau_{a,c} = 2.28 \pm 0.13$ ms, $n = 50$, $\tau_{a,GI} = 1.86 \pm 0.09$ ms, $n = 48$, $\tau_{a,GII} = 1.79 \pm 0.08$ ms, $n = 40$, $p = 0.001$).

To study the inactivation kinetics of Kv1.3 current two-second-long step pulses to +40 mV from a holding of −120 mV were

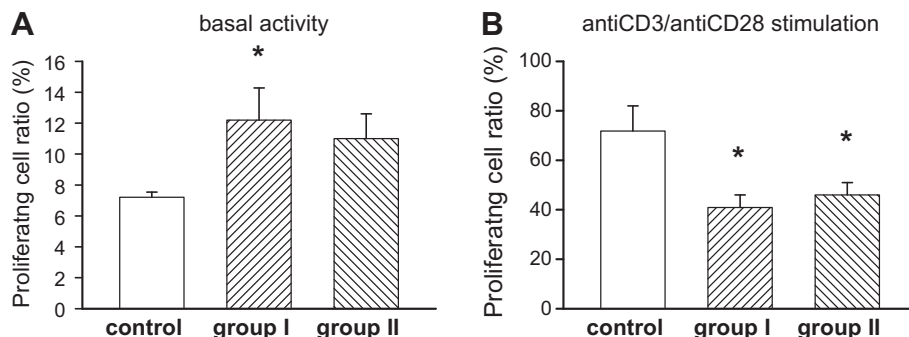


Fig. 1. Proliferation of T cells altered in hypercholesterolemia. (A) Basal lymphocyte activity in control, group I and group II cells. (B) Ratio of proliferating lymphocytes after antiCD3/antiCD28 stimulation determined by CFSE assay for different patient groups. The error bars represent SEM for seven individuals.

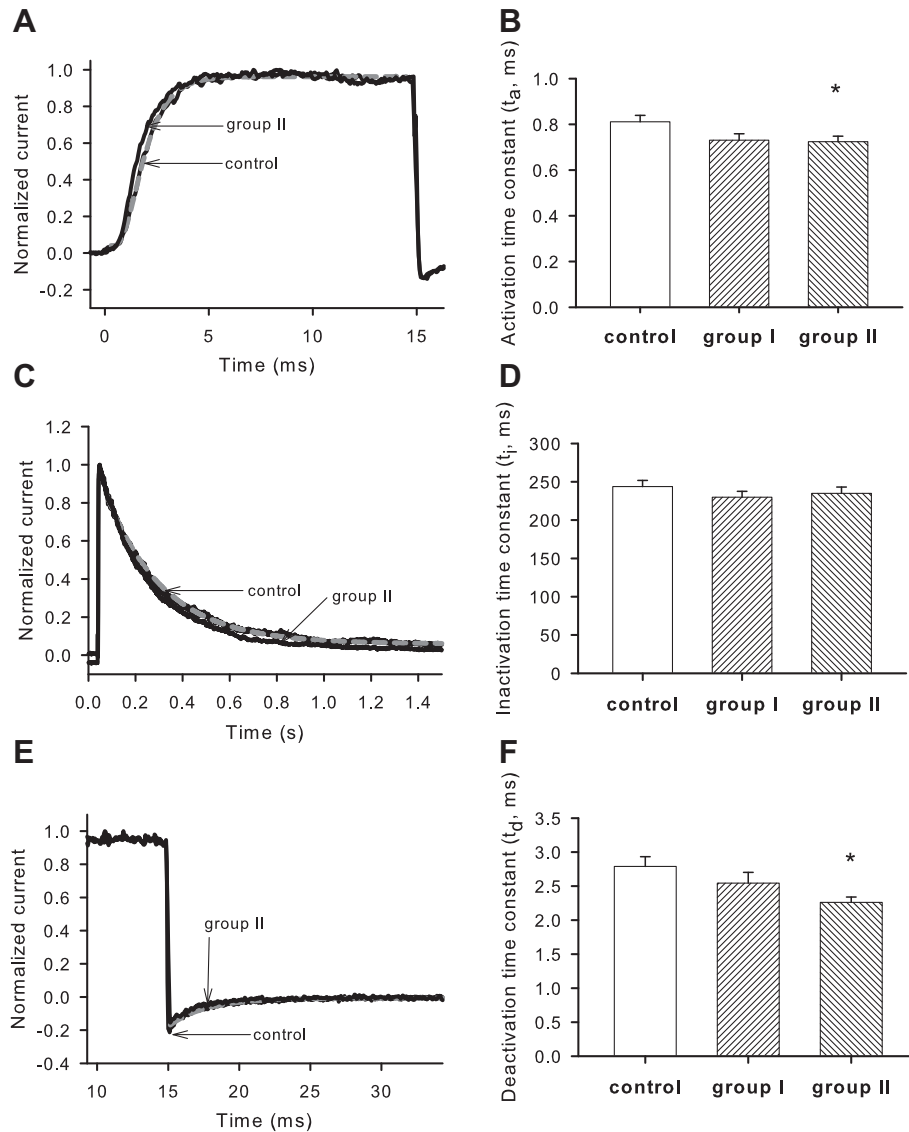


Fig. 2. Kinetic properties of whole-cell Kv1.3 current in control and hypercholesterolemic cells. (A) To study the activation kinetics of the whole-cell Kv1.3 current depolarizing steps to +50 mV were applied for 15 ms from a holding potential of –120 mV. To reduce the capacitance and leak artifacts on-line leak subtraction was applied using a p/5 protocol. Arrows indicate typical current traces for a control and a group II T cell. For clear visibility the best fitting curve obtained using Hodgkin-Huxley model is indicated for the control cell only (dashed line, $\tau_{a,c} = 0.88$ ms and $\tau_{a,II} = 0.77$ ms). (B) Average activation time constant for three groups are shown, error bars represent SEM ($n \geq 41$). (C) To determine the inactivation kinetics for Kv1.3 currents depolarization steps to +40 mV lasting for 1.5 or 2 s were delivered to the cells from a holding potential of –120 mV. Two representative current recordings are illustrated (control, group II, indicated by arrows) along with the best fitting single exponential decaying curve to the decaying part of the control trace ($\tau_{i,c} = 247$ ms and $\tau_{i,II} = 242$ ms). (D) Average inactivation time constant for three groups are shown, error bars represent SEM ($n \geq 35$). (E) To determine the deactivation kinetics of the Kv1.3 current cells were first depolarized to +50 mV for 15 ms to open all the channels and then instantaneously repolarized to –120 mV to obtain the tail currents. Two typical tail currents recorded –120 mV are shown (control, group II, indicated by arrows) along with the best fitting single exponential function for the control trace (dashed line, $\tau_{d,c} = 2.4$ ms and $\tau_{d,II} = 2.1$ ms). (F) Average deactivation time constant for three groups are shown, error bars represent SEM ($n \geq 35$).

applied to the cells in every 60 s as illustrated in Fig. 2C. 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 ($I(t) = I_0 \times \exp(-t/\tau_{i,x}) + C$, 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 ($\tau_{i,c} = 243.8 \pm 8.0$ ms, $n = 41$;

$\tau_{i,GI} = 229.3 \pm 6.9$ ms, $n = 48$; $\tau_{i,GII} = 234.9 \pm 8.2$ ms, $n = 35$, $p = 0.384$, Fig. 2D).

Furthermore, to learn the closing (deactivation) kinetics of Kv1.3 channels the tail-currents at a negative membrane potential were also analyzed. Tail-currents were recorded upon a repolarization step to –120 mV, which was preceded by a 15-ms-depolarization to +50 mV from a holding of –120 mV (Fig. 2E). The decay of the inward tail-current was fitted with single exponential function (as for inactivation), which resulted in the deactivation time constant for Kv1.3 current. The deactivation accelerated in hypercholesterolemic state, the deactivation time constant of the current reduced significantly in group II compared to control ($\tau_{d,c} = 2.79 \pm 0.14$ ms, $n = 35$; $\tau_{d,GI} = 2.54 \pm 0.16$ ms, $n = 45$; $\tau_{d,GII} = 2.26 \pm 0.08$ ms, $n = 39$, $p = 0.028$, Fig. 2F).

To evaluate the effect of age we checked correlation between age and Kv1.3 kinetic parameters. We could not see any significant correlation (data not shown), which rules out that the age difference caused the kinetic changes between control and Group II.

3.5. Steady-state parameters of activation

Since the kinetic parameters of the Kv1.3 gating were influenced by the elevated serum cholesterol level we also studied the equilibrium parameters of the activation. The voltage-dependence of the normalized whole-cell conductance (G_N) was used to estimate the ratio of the pools of closed and open channels at the defined test potential. Fig. 3A illustrates the normalized conductance-test potential relationship for a healthy and a group II T cell along with the best-fitting Boltzmann functions (see figure legend also for description). Neither the midpoint of voltage-dependence of steady-state activation ($V_{1/2,c} = -24.9 \pm 0.87$ mV, $n = 26$, $V_{1/2,GI} = -26.5 \pm 1.0$ mV, $n = 38$, $V_{1/2,GII} = -26.1 \pm 0.9$ mV, $n = 27$, Fig. 3B) nor the slope factor of steady-state activation ($s_c = 10.8 \pm 0.35$, $n = 26$, $s_{GI} = 10.4 \pm 0.22$, $n = 38$, $s_{GII} = 10.0 \pm 0.26$, $n = 27$, Fig. 3B) was different among the control, group I and group II T cells ($p = 0.435$, $p = 0.186$, respectively).

3.6. Cholesterol content of T cells in hypercholesterolemia

As the electrophysiological data demonstrated changes in the gating of the Kv1.3 channels in hypercholesterolemic conditions we determined the cholesterol content of lymphocytes from different groups. A commercially available cholesterol assay was applied (see materials and methods). Furthermore, the concentration of cholesterol was normalized to the absorbance detected at 280 nm, which is a good estimator of the amount of the cells in a sample (each sample contained app. 10^6 cells/ml). To validate our technique, we introduced a positive control: normocholesterolaemic lymphocytes were loaded with cholesterol complexed to methyl- β -cyclodextrin (M β CD/C) at concentration of 1.5 mg/ml. As Fig. 4 shows the cholesterol concentration of samples from three different hypercholesterolaemic patients of group II is higher than the control value (control: 0.66 ± 0.009 μ g/ml, $n = 3$, group II: 0.91 ± 0.07 μ g/ml, $n = 3$, $p < 0.001$). However, cholesterol loading using 1.5 mg/ml M β CD/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 M β CD/C: 1.53 ± 0.03 μ g/ml, $n = 3$, $p < 0.001$).

4. Discussion

In this study we reported that proliferation capacity of peripheral blood T lymphocytes from hypercholesterolemic patients is different from that of the healthy cells. To reveal the possible mechanisms behind the altered activation behavior we inspected whether functional changes of Kv1.3 channels are associated with this observation. Using patch-clamp technique we studied expression level, kinetic and equilibrium parameters of the gating of Kv1.3 channel and found that (i) neither the whole-cell peak current of Kv1.3 channels nor the current density were changed in hypercholesterolemic state (ii) activation kinetics of the current at 0 mV was significantly faster for group I and II, while at +50 mV for group II than in control cells; (iii) inactivation and deactivation of the channel speed up in hypercholesterolemia but only deactivation kinetics of group II was significant (iv) midpoint and slope of the voltage-dependence of steady-state activation were the same for all groups.

The participation of T cells in atherosclerosis is widely accepted: T_H1 cells can exacerbate the atherosclerotic status, while T_{reg} lymphocytes in the atherosclerotic lesions downregulate the effector mechanism and cytokine production of T_H1 cells. On the other hand T_H2 subset can have either proatherosclerotic or protective effect but it is not fully clear yet. It was also shown that autoantigens are produced against different chemical forms of LDL and hsp60; and also native LDL reactive, peripheral T cell subclones could be isolated from hyperlipidemic mice. Dendritic cells, which take up oxidized LDL (oxLDL) through endocytosis can also trigger T cells proliferation in atherosclerosis. Kv1.3 channels, which are present in every human T cell subsets are key participant in regulation of intracellular Ca²⁺ level [34–37]. Their role in the Ca²⁺-dependent activation upon antigen stimulation via TCR/CD3 receptor complex has been firmly established by several groups; hence any modification or loss of their function can lead to the cease of the T cell clonal expansion [35,38]. For example, it has been described that only a “simple” immobilization of Kv1.3 channels in T lymphocytes cells was able to modify the Ca²⁺-signaling upon formation immunological synapse [39]. A study on correlation of Kv1.3 function and Ca²⁺ signaling reported that in type 1 diabetes the faster onset of maximum Ca²⁺ level and reactivity of T cells was due to the increased activity of the ion channel [40]. Based on these facts, we think that higher basal proliferative rate of hypercholesterolemic lymphocytes can be attributed to presence of an activated, oxidized and native LDL and Hsp 60 specific T cell subset,

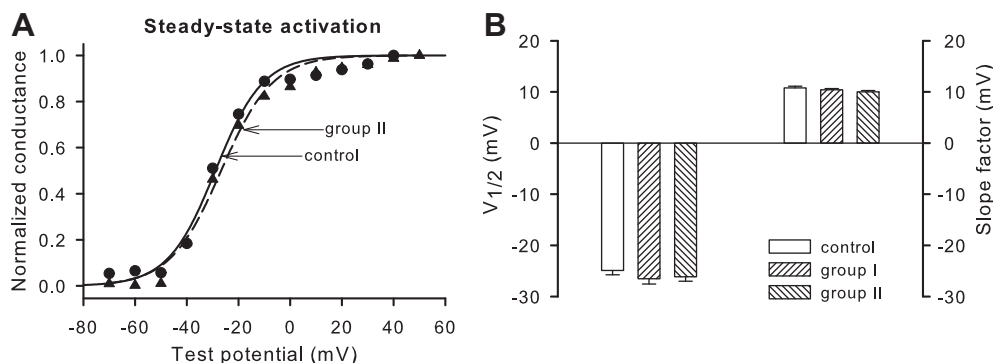


Fig. 3. Steady-state parameters of activation for Kv1.3 channels. (A) Voltage-dependence of steady-state activation of the Kv1.3 channels in a control (filled circle) and a group II (filled triangle) cell. The cells were clamped at holding potential of -120 mV and depolarized to a set of test potentials ranging from -70 to $+40$ mV in 10 mV increments at every 30 s. Peak whole-cell conductance ($G(V)$) at a given test potential was derived from the peak current (I_p) at test potential V and the K⁺ reversal potential (E_r) using $G(V) = I_p / (V - E_r)$. The $G(V)$ values were normalized for the maximum conductance and plotted as a function of test potential along with the best fit Boltzmann-function (solid line; control, dashed line; group II normalized conductance (G_N) = $1 / (1 + \exp[-(V - V_{1/2,a})/k_c])$, where V is the test potential, $V_{1/2,a}$ is the midpoint and k_c is the slope of the function). The slope factor and the midpoint are 9.2 and -28.9 mV for the control cell, and 9.9 and -26.7 mV for the group II cell. (B) The average of the midpoint of the steady-state activation (left axis) and the slope factor (right axis) for the three groups. Error bars indicate SEM ($n \geq 23$).

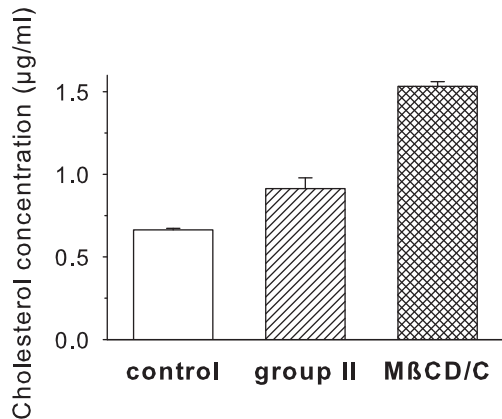


Fig. 4. Cholesterol concentration in hypercholesterolemic T cells. The cholesterol concentration in lymphocytes was determined by means of Amplex Red cholesterol assay kit (see materials and methods). The cholesterol concentration for control (empty bar), group II (hatched bar) and 1.5 mg/ml MβCD/C cholesterol loaded samples (cross-hatched bar) was measured in triplicates, normalized to the absorbance measured at 280 nm, and averaged for $n \geq 3$, the error bars represent SEM.

which can go through the steps of proliferation in the absence of specific autoantigens.

During *in vivo* activation of T cells engaged to an antigen presenting cell (APC) several proteins – including Kv1.3, TCR/CD3 – redistribute into the immunological synapse (IS), where several phosphorylation events initiate signaling of activation. However, treatment of T cells' membrane with oxysterols results in a less efficient signaling domain formation, phosphorylated proteins do not reside in the cell membrane and actin remodeling is impaired at the IS [41]. Furthermore, it is supposed that Kv1.3 has a major role in clustering and organizing several proteins at the synapse, as well as maintaining negative membrane potential at the IS to ensure Ca^{2+} influx through CRAC channels [42]. Hence, the decreased fraction of divided T cells upon CD3/CD28 challenging under hyperlipidemic conditions may verify the scenario that Kv1.3 channels and TCR/CD3 receptors have a reduced activity since the serum oxLDL leads to the collapse of raft system in their membrane.

Several studies, which demonstrated the effect of *in vitro* manipulation the cholesterol content on the function of Kv1.3 channels, are rather controversial. We previously reported that the activation and inactivation of Kv1.3 channels are slower and the voltage-dependence of steady-state activation was rightward-shifted in human T cells loaded with cholesterol [25]. A study by Pottosin et al. reported that acute application of MβCD, which causes depletion of the membrane cholesterol, resulted in the slowing of the activation kinetics of the Kv1.3 current in Jurkat cells [43]. Moreover, independent research groups demonstrated that Kv1.3 channels are targeted to lipid rafts in T cells, and their function highly depends on the composition of these microdomains [26,44,45]. In a recent paper Levitan et al. reviewed that oxLDLs modify the packing of lipids in cholesterol-rich membrane domains without changing the cholesterol level: incorporation of oxysterols into the membrane can disrupt the lipid rafts [46]. Consequently, we suppose that faster kinetics of Kv1.3 current in hypercholesterolemic condition can be attributed to the redistribution of the channels into cholesterol-poor/non raft domains [46]. In these disordered domains of the membrane the fluidity is higher compared to the raft compartments so the molecular motions can speed up. Furthermore, the subunit structure of the channel protein can account for the lesser amount of change in the inactivation and deactivation compared to activation: both previous

processes occur at that segment of channel, which do not directly interface the surrounding lipid environment, just the intramolecular interactions affect their movement [44,45].

Previously it was described that proper gating of ion channels requires the existence of annular lipids firmly attached to the channel protein [47,48]. Lu's laboratory also showed that enzymatic modification of sphingomyelins, which is also a main constituent of lipid rafts, resulted in the reduction of whole-cell Kv1.3 current in oocytes but no change in the midpoint and slope of the activation [49,50]. Here we reported that steady-state parameters of Kv1.3 activation do not differ in control and patient's groups. Consequently, we speculate that putative translocation of Kv1.3 channels into the non-raft region due to oxLDL uptake does not interfere the annular lipid-channel interaction, thus no shift in the G–V relationship can be detected.

Furthermore, the current density in each group was the same, though an increase in the expression level of Kv1.3 upon is expected during activation. A possible explanation is that only a small subset of T cells, which are committed to e.g. LDL autoantibodies are activated and these may have higher number of Kv1.3 in the membrane. Second, we mentioned above the redistribution of Kv1.3 outside rafts can also influence the activity of the channel: even though the higher number of channels is in the membrane their function is hindered by the lipid environment.

We also monitored the cholesterol content of group II cells, which was approximately 1.5-fold higher than in the normcholesterolemic cells but it was far below that of the 1.5 mg/ml MβCD/C treated T cells (Fig. 4). Moreover, we are certain that native cholesterol molecules do not only partition into the plasma membrane but into the intracellular lipid vesicles, bilayers unlike the cells incubated with MβCD/C. Therefore the serum cholesterol level is likely to have a smaller effect on the plasma membrane cholesterol level and its physical-chemical properties such as viscosity.

In summary we demonstrated that hypercholesterolemia in human affect the proliferative ability of T cells, which may occur due to the presence of inflammatory conditions in atherosclerosis (autoreactive T cells) and the modification of the raft system. We suppose that instead of the direct effect of serum cholesterol on the lipid rafts of plasma membrane the raft-rupturing behavior of various oxLDL species is responsible for the changes of Kv1.3 function.

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