

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Characterization of Kv1.3 K⁺ ion channel expression and gating in T lymphocytes

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The Examination takes place at the library of the Department of Physiology,
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1 INTRODUCTION

1.1 Mechanisms of T cell activation

Clonal proliferation of lymphocytes brought about by binding of specific antigens is one of the fundamental events in the proper function of the immune system. Proliferation is initiated following activation of the T cell receptor/CD3 complex (TCR/CD3), when an antigen-presenting cell presents a specific peptide fragment of an antigen to the T lymphocyte by displaying it with its major histocompatibility complex (MHC), thereby forming an MHC–peptide fragment–TCR/CD3 complex. This first step in the signalling pathway then leads to the activation of various kinases and phospholipase C- γ (PLC γ). The activation of PLC γ initiates two distinct signalling pathways: phosphatidylinositol 4,5-bisphosphate (PIP₂) is cleaved into 1,2-diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). DAG in turn activates protein kinase C enzymes (PKC) and enhances the nuclear translocation of activator protein-1 (AP-1). Besides protein kinases, a biphasic rise in cytosolic Ca²⁺ concentration is another important step in T cell activation. This process is triggered by the activation of IP₃ receptors located in the endoplasmic reticulum (ER) membrane upon binding of IP₃ that diffuses freely in the cytosol; this results in Ca²⁺ release from the ER that elevates resting cytosolic Ca²⁺ levels in activated lymphocytes. However, this cytosolic Ca²⁺ component is insufficient for proper gene expression control. The extracellular space represents another important source for elevated intracellular Ca²⁺ concentration. Depletion of internal Ca²⁺ stores triggers the activation of the stromal interaction molecule 1 (STIM1), which then migrate to specific sites of the ER membrane where they can activate Orai1 molecules located in the plasma membrane; these latter molecules form the pore of Ca²⁺ release-activated Ca²⁺ (CRAC) channels. Sustained, oscillatory rise in intracellular Ca²⁺ concentration then results in the calmodulin-dependent activation of calcineurin; this in turn promotes nuclear translocation of the transcription factor NF-AT, which enhances transcription of

Interleukin-2 (IL-2) and initiates cellular proliferation by close co-operation with other signalling pathways. IL-2 expression is indispensable to further (antigen-independent) steps of T cell activation.

1.2 The immunological synapse

The first step of *in vivo* T lymphocyte activation is antigen presentation that manifests in the formation of the immunological synapse (IS) between the antigen bound to the MHC protein of the antigen-presenting cell and the TCR/CD3 complex. The IS represents the interaction surface between the antigen-presenting cell and the T cell that recognises the antigen. Formation of the IS leads to reorganisation and segregation of plasma membrane proteins into subcellular membrane domains in both cells, thereby establishing the supramolecular activation complex (SMAC). Abraham Kupfer was the first to define the central TCR complex between T and B cell conjugates as a central supramolecular activation cluster (cSMAC). Furthermore, cSMAC can be characterised as having two distinct regions; an internal and an external cSMAC. In the former one, large amounts of TCR; whereas in the latter one, CD28 and PKC- Θ membrane and cytosolic proteins dominate the T cell side of the immunological synapse. Besides the cSMAC region determined by the CD3 receptor, the peripheral SMAC (pSMAC) also contributes to stabilise contact between the two cells that establish the immunological synapse. The central supramolecular unit is surrounded by talin and a ring formed by the lymphocyte function-associated antigen 1 (LFA-1) and the intercellular adhesion molecule 1 (ICAM-1), collectively referred to as the peripheral supramolecular activation cluster. LFA-1 and ICAM-1 initially function as adhesion molecules, facilitating the T cell–APC, as well as the TCR–MHC-peptide fragment bonds to form. LFA-1 that binds to ICAM-1 integrates into the pSMAC network.

The above-mentioned regions are all made of several thousands of molecules that are bound together by non-covalent bonds in this functional

network. The proper spatial orientation of the proteins ensures that the activation of the TCR/CD3 complex should be effective by the molecular proximity of protein kinases. Later on, Kupfer also defined a distal supramolecular activation cluster (dSMAC) as the outermost component of the complex, in which CD45 is one of the central components. The distal pole complex was described by Burkhardt; this complex may be important in separating specific negative regulators such as SHP-1.

Furthermore, besides adhesion proteins, co-stimulatory proteins such as CD28 or CD2 (the latter also has adhesive functions) also play crucial roles in the formation of TCR microclusters.

1.3 Characteristics of the Kv1.3 ion channel

1.3.1 Biophysical characteristics of the Kv1.3 ion channel

The Kv1.3 ion channel belongs to the *Shaker* family of K⁺ channels. *Shaker* ion channels are almost symmetric tetramers formed by four identical subunits. All subunits are comprised of approx. 500 amino acids; non-covalent bonds between subunits hold the tetramer together. Each subunit consists of six transmembrane alpha-helical segments that are connected to each other by extra- and intracellular loops. These segments and loops have well-determined roles in channel functions. The first four segments are involved in channel opening – the fourth segment is especially important during activation owing to its positively charged residues, – while the extracellular loop between segments 5 and 6 forms the pore. A short sequence formed by four residues (GYGD) is located in this segment, which is responsible for the K⁺ selectivity of the channel; moreover, ion channel blockers that bind inside the pore also interact with residues in this region.

Similar to *Shaker* ion channels, Kv1.3 channels also open when the membrane is depolarised; this is achieved by a cooperative mechanism of the four subunits. Formed by the strongly positively charged S4 and the net

negatively charged S3 helices, the voltage sensor is sensitive to changes of the membrane potential. The activation threshold voltage of Kv1.3 ion channels is approx. -50 mV, which aligns with the resting membrane potential of T cells. Above the activation threshold, the open probability of the ion channel rises rapidly with membrane depolarisation. The voltage-dependence of the steady-state activation is characterised by a sigmoidal Boltzmann function; it reaches the saturation value around $+20 - +30$ mV, when the open probability of the ion channel is 1. The steady-state inactivation of Kv1.3 ion channels is not complete at the resting membrane potential of T cells. The voltage-dependence of steady-state activation and inactivation determines a membrane potential window in which Kv1.3 channels are active. This window aligns with the resting membrane potential of lymphocytes; therefore the K^+ flux across Kv1.3 ion channels may contribute to setting the diffusion membrane potential.

Following rapid activation, *Shaker* K^+ channels enter a non-conducting, inactive state when the membrane is depolarised; this can occur by either the N- or C-type inactivation mechanism. The fast N-type inactivation is reached within a few milliseconds; a short peptide with more positively charged residues at the intracellular N-terminus of the protein will act as a blocker by binding into the cytoplasmic side of the channel, thereby blocking the pore. Nonetheless, many *Shaker* K^+ channels, including Kv1.3 lacks this inactivation unit; therefore, the slow C-type mechanism is responsible for their inactivation, which occurs within a few hundred milliseconds or sometimes several seconds.

C-type inactivation is probably accomplished by a conformational change brought about by a complex mechanism that involves the selectivity filter and the extracellular side of the pore. The C-type inactivation is sensitive to external cations and the amino acid composition of the channel pore. This process is inhibited by high extracellular K^+ concentration; it is insensitive to intracellular TEA, but it can be inhibited by TEA applied extracellularly. The voltage sensor may also be involved in the inactivation by undergoing a conformational

change.

The slow inactivation of *Shaker* ion channels comprises two sequential processes. Initially, the outer pore of the channel becomes narrower that prevents ion flow, followed by a much slower conformational change in the selectivity filter that stabilises the voltage sensor and the pore. Inactivation reduces the number of available K^+ channels for membrane potential control. Inactivated Kv1.3 channels require a relatively long period (50 s) to recover into their resting (closed) state even if the membrane potential is approx. -120 mV; therefore, activated Kv1.3 channels can allow for a considerable K^+ flux before entering a non-conductive inactive state. The above characteristics of Kv1.3 ion channels, as well as the particularly high resistance of the lymphocyte plasma membrane (10–20 $G\Omega$) can be accounted for the fact that relatively few (200–300) ion channels are sufficient for maintaining the lymphocyte membrane potential.

1.3.2 Postsynthetic modification of the Kv1.3 ion channel

Besides inhibitors, targeted manipulation of biophysical parameters that characterise the gating of ion channels can also be used to modulate the activity of K^+ channels. This can be achieved by protein-protein interactions, including regulation of ion channels by protein kinases.

PKA and PKC were reported to be negative regulators of Kv1.3 ion channel activity in Jurkat T cells; moreover, PKC-dependent phosphorylation is a prerequisite to regulation by PKA. By contrast, Kv1.3 ion channel phosphorylation by PKA and PKC is a positive regulator by modulating the voltage-dependence of steady-state activation and inactivation. PKC-dependent regulation (phosphorylation on Ser/Thr residues) of Kv1.3 ion channel activity has been confirmed in Jurkat cells; however, the nature of PKC and PKA-mediated regulation was largely dependent on ambient proteins that form the native environment of the ion channel.

Furthermore, tyrosine kinases were found to be negative regulators of Kv1.3-mediated currents. In T cells, however, signalling is mediated via non-receptor tyrosine kinases that are upregulated following TCR/CD3 activation; regulation of Kv1.3 by these kinases is therefore of more physiological relevance. Co-transfection of non-receptor protein kinases (v-src) and Kv1.3 ion channels into HEK-293 cells resulted in enhanced tyrosine phosphorylation of the ion channel protein, which manifested in a macroscopic current decrease in “cell-attach” patch configuration. Application of tyrosine phosphatase inhibitors (pervanadate) resulted in enhanced phosphorylation of Kv1.3 by endogenous kinases, which also resulted in reduced current amplitude.

Furthermore, results of several biochemical and biophysical experiments implicate that besides the well-known molecules Kv1.3 ion channels are also key components in the signalling complex formed during the IS. It is known that Kv1.3 ion channels are regulated by p56^{lck} kinase and that Kv1.3 and p56^{lck} are connected to each other via the hDlg (SAP97) adapter protein; moreover, association of the accessory subunit (Kvβ1.2) of Kv1.3 channels to PKC via other adapter proteins (ZIP-1, ZIP-2) also supports this theory. The hypothesis that there may be a connection between Kv1.3 ion channels and other molecules of the immunological synapse is further supported by the fact that these proteins were found to associate to cholesterol and sphingomyelin-rich membrane microdomains (also known as lipid rafts) that dynamically reorganise following activation of the TCR/CD3 complex.

Based on the above, it can be assumed that reversible phosphorylation modifies the function of the Kv1.3 ion channel, and that accumulation in the IS influences the effectiveness of signalling pathways.

1.4 Regulatory T cells and autoimmunity

Regulatory T cells (Treg) are CD4⁺ CD25⁺ cells that play important role in maintaining the immune tolerance for own tissues and in suppression of

autoimmune processes. The number and activity of Tregs may change in certain physiological conditions. Tregs have a reduced suppressing effect on T cell mediated immune responses in multiple sclerosis (MS) and type 1 diabetes and other autoimmune diseases, whereas elevated Treg numbers and their enhanced functioning have been associated with the development of various types of cancers.

Initially, Tregs were identified solely by the high expression level of the interleukin-2 receptor α -chain (CD25), but later the expression of the transcription factor FoxP3 was considered as the most specific marker of T cells with regulatory function. Being an intracellular marker, labeling for FoxP3 requires permeabilization of the cells which prevents many physiological investigations. Recently, however, it was found that CD127, which is part of the heterodimeric IL-7 receptor, is down-regulated in Tregs and CD25^{hi}CD127^{lo/-} expression showed very high correlation with FoxP3 expression. CD127 is down-regulated in all human T cells after activation, but in the majority of effector and memory T cells it is re-expressed, while FoxP3⁺ T cells remain CD127^{lo/-}. CD4⁺CD25^{hi}CD127^{lo/-} T cells isolated by flow cytometric cell sorting demonstrated the hallmark features of Tregs, namely the suppression of the proliferative response of alloreactive T cells in a mixed lymphocyte response while themselves being anergic to the same stimuli.

Due to their central role in immune control and their altered functioning in cancer and autoimmune diseases Tregs are now emerging as potential targets for therapeutic approaches. Treatments that could selectively stimulate the regulatory functions of Tregs in autoimmune conditions or suppress them in cancer would be of enormous value.

1.5 Kv1.3 channel blockers may cause specific immunosuppression

Chandy and colleagues reported that patients with the above-mentioned conditions and in activated effector memory T cells (T_{EM}) isolated from

pathological tissue environments Kv1.3 expression is significantly higher than in naïve and central memory T cells (T_{CM}) (~1,500/cell). By contrast, the number of IKCa1 ion channels is much smaller in activated T_{EM} cells than in the latter two cell type. Furthermore, in case of naïve ($CCR7^+CD45RA^+$) and T_{CM} cells ($CCR7^+CD45RA^-$), mitogen-activated proliferation results in a mild (~250/cell $\rightarrow$ 400/cell) elevation of Kv1.3 channel expression, but at the same time causes significantly (8–10/cell $\rightarrow$ 500/cell) higher IKCa1 expression.

All in all, the membrane potential of T_{EM} cells, as well as all of the resting T cells mentioned above is primarily determined by Kv1.3 ion channels, whereas IKCa1 is the predominant ion channel in the naïve and T_{CM} subpopulations. High-affinity inhibitors of Kv1.3 (*e.g.* ShK toxin, $IC_{50} \sim 100$ pM) specifically block the proliferation of T_{EM} cells, since enhanced IKCa1 expression in resting (naïve) and T_{CM} cells rescues these cells from the Kv1.3 blocker-mediated inhibition of proliferation. By selectively blocking the Kv1.3 ion channel, the activation of T_{EM} cells can be inhibited, thereby immunosuppression can be achieved.

2 AIMS

The experimental work presented here has two main scopes:

- A. Study the role of Kv1.3 ion channels in the immunological synapse
- B. Analyse the ion channel expression pattern of regulatory T cells isolated from patients with sclerosis multiplex

The aims of our research were as follows:

A.

1. Biophysical characterisation (steady-state and kinetic parameters) and pharmacological studies of K^+ ionic currents in murine D10 T_H2 cell line by applying a specific ion channel inhibitor.
2. Setting up an antigen-presenting cell (APC) – T cell model system to study the immunological synapse.
3. Investigation of Kv1.3 ion channels in the immunological synapse by immunofluorescent labelling.
4. Comparison of steady-state and kinetic characteristics of K^+ currents in D10 cells under control conditions and in immunological synapse.
5. Determination of factors that regulate ion channel translocation by applying protein kinase inhibitors and to study the functional consequences of Kv1.3 channel protein accumulation in the immunological synapse.

B.

We also aimed at studying the ion channel expression pattern in regulatory T cells (T_{reg}); we aimed to establish whether this special T cell subpopulation is characterised by a special ion channel expression pattern, and whether there is a difference between the ion channel expression pattern of T_{reg} and naïve T cells in patients with autoimmune disorders and in healthy individuals.

3 MATERIALS AND METHODS

3.1 Cells and conjugate formation

We used the conalbumin specific D10-PKC- θ -GFP T_H2 cell line that expresses the wild-type form of PKC- θ with a GFP as antigen recognizing and CH12-LX cells as antigen presenting cells. CH12-LX cells were pulsed overnight with 500 μ g/ml conalbumin. Cell conjugates were formed by mixing CH12-LX cells with D10 cells at a 1:1 cell ratio. The IS could be identified since the GFP tagged PKC- θ enzymes accumulated in the contact region of the two cells.

We used HEK-tsA cells as expression system for investigating the possible presence of the two-pore channels.

Human peripheral lymphocytes were isolated from 6 patients of multiple sclerosis and 9 healthy objects.

3.2 Immunohistochemistry and confocal laser scanning microscopy

After formation of the synapse, D10 cells of the conjugates were fixed on poly-L-lysine-covered coverslip and fixed with 3.75% paraformaldehyde. D10 cells were incubated overnight with anti-Kv1.3 (against its extracellular epitope), then with Cy3-conjugated secondary antibodies for 1 hour. Microscope images were taken using the Olympus FW1000 confocal laser scanning microscope. Excitation of the GFP was at 488 nm with Argon ion laser, while Cy3 was excited at 543 nm with HeNe laser. Detection filters were the following: 515/15 BandPass (GFP) and 605/50 BandPass (Cy3).

3.3 Flow cytometry

Human peripheral lymphocytes were labeled with anti-CD4-FITC, anti-CD127-PE and anti-CD25-APC antibodies. Cell sorting was performed by FACSVantage SE DiVa equipment. Excitation of the fluorophores was at 488 nm with Argon ion laser, at 532 nm with diode pumped solid-state laser and at

633-nm HeNe laser. Detection filters were the following: 530/30 BandPass (FITC) and 650 LongPass (APC).

3.3.1 Separation of CD4⁺ regulatory and naïve T cells

Human T cells from the peripheral blood of healthy volunteers and MS patients were sorted for patch-clamp measurements by flow cytometry. Cells were distinguished based on the expression levels of CD4, CD25 and CD127 cell surface markers, such that CD4⁺CD25^{hi}CD127^{lo} cells were considered as Tregs, while CD4⁺CD25^{lo}CD127^{hi} cells were considered as naïve cells. Since patch-clamping is performed on individual cells and the number of cells required for patch-clamping is much lower than the number of sorted cells, the priority during sorting was on high purity rather than high yield.

3.4 Reagents

Tetraethylammonium (TEA), H89 and GF109203X were purchased from Sigma–Aldrich Ltd., Hungary. Damnacanthal was bought from Merck Ltd., Hungary. Margatoxin (MgTx) was ordered from Alomone Labs., Israel.

3.5 Electrophysiology

Patch-clamp measurements were carried out in whole-cell or in outside-out configuration in voltage clamp mode using Axopatch-200B and Axopatch-200A amplifiers. Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries in two stages and fire-polished to give electrodes of 2–3 MΩ resistance in the bath.

The base of the bath solution was 145 mM NaCl, the base of the pipette solution was 140 mM KF.

3.6 Data analysis

The whole-cell conductance was defined by $G = I_{\max} / (V - E_{\text{rev}})$, where $I_{\max}$ is the measured peak current at V test potential, E_{rev} is the reversal potential. The number of the channels in the membrane was calculated by the ratio of the whole-cell conductance and the conductance of a channel individually.

Current density was defined by the ratio of current amplitude and capacitance.

Statistical comparisons were made using t-test, (the level of significance was 0.05) or Mann-Whitney rank sum test (MWRST). For all experiments, the standard error of the mean (SEM) is reported for at least $n = 4$ or as it is indicated.

4 RESULTS

4.1 Kv1.3 in the immunological synapse

4.1.1 Biophysical and pharmacological characterization of whole-cell currents of D10 cells

We characterized voltage-gated potassium currents thoroughly in D10 cells. Current traces recorded in a D10 cell upon short, 15 ms depolarizing steps used to reveal kinetic properties of activation. The activation of the potassium current could be fitted 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 one cell was given as the average of time constants obtained for depolarizations in a sequence containing 3 pulses repeated at every 15 s ($\tau_{a,c} = 1.36 \pm 0.06$ ms, $n = 18$).

To elucidate the inactivation time constant of K^+ current 1.5 or 2-s-long depolarizations to $+40$ mV were delivered to the cells and the decaying part of the registered curves was fitted with a single exponential function for each cell ($I(t) = I_0 \times \exp(-t/\tau_{in}) + C$, I_0 : amplitude of current, τ_{in} : inactivation time constant, C : steady-state value of whole-cell current at the end of the pulse). The inactivation kinetics of one cell was defined as for activation, except the depolarizations which were separated by 60 s. The average inactivation time constant in D10 cells was 365 ± 27 ms ($n = 17$).

Furthermore, the voltage-dependence of normalized whole-cell conductance (G_N , G_N – V curve), which describes the equilibrium distribution of the ion channels between the closed and open states at a given membrane potential, were determined to obtain the steady-state parameters of activation. Normalized conductance was plotted as a function of test potential cell with the superimposed Boltzmann function ($V_{1/2,c} = -26.4 \pm 1.5$ mV, $k = 7.6 \pm 0.5$ mV, $n = 12$). Comparing the kinetic and steady-state parameters of activation and

inactivation to those reported earlier for K^+ current in murine T cell lines we supposed that the current of Kv1.3 channels had been detected in D10 cells.

However, some murine T lymphocyte lines express Kv3.1 channels, which may also contribute to the whole-cell current in D10 cells. Nonetheless, the pharmacology of Kv1.3 and Kv3.1 channels is very hence in the next course of experiments we performed pharmacological characterization of the K^+ current.

A general inhibitor of K^+ channels tetraethylammonium (TEA) and a high-affinity blocker of Kv1.3 channel margatoxin (MgTx) were applied in 10 mM and 100 pM concentration, respectively. The inhibitory effect of 50 pM MgTx: the peak current measured in normal bath solution dropped to 22% of the control value when the cell was perfused with peptide toxin. Two-parameter Hill equation was fitted to the dose–response curves, which resulted in a K_d value of 13.4 mM for TEA and 16.8 pM for MgTx. Altogether, the biophysical and pharmacological properties of the measured K^+ currents clearly prove that D10 cells dominantly express Kv1.3 channels in their cell membrane.

4.1.2 The lack of TASK channels in D10 cells

In order to exclude the presence of TASK-1 and TASK-3 channels, we focused our experiments on these possibly expressing two-pore channels.

The TASK current is voltage-independent, and its current–voltage relationship is very similar to that of the Ca^{2+} -activated K^+ channel (IKCa1) in lymphocytes. Since we applied a KCl-based, Ca^{2+} -free pipette filling solution the contribution of IKCa1 channels to the whole-cell current was negligible. To eliminate Kv1.3 channels we applied 0 mV holding potential (at which Kv1.3 channels are inactivated at steady-state), 100 pM MgTx and a double-ramp protocol indicates that the reversal potential of the whole-cell current (no functional Kv1.3 and IKCa1) is at ~ -4 mV, which is much more positive than the one expected in the presence of TASK channels (~ -85 mV, based on the

composition of the solutions, using the Nernst equation). Moreover, no or slight rightward shift in the current–voltage relationship could be seen upon perfusion with high K^+ bath solution. Based on this, we can exclude the expression of a significant number of TASK channels in D10 cells.

4.1.3 The insensitivity of two-pore K^+ channels to Margatoxin (MgTx)

mTASK-1 and h-TASK3 plasmid DNAs were transfected into HEK-tsA cells. We applied 100 pM MgTx using voltage ramp protocol at 0 mV holding potential. MgTx in this concentration did not affect the current. This result strongly justifies that K^+ channel expression in D10 cells is dominated by Kv1.3 and the contribution of TASK-1 or TASK-3 to the whole-cell current is negligible.

4.1.4 The effect of TASK blockers on Kv1.3 currents

Anandamide (AEA) was demonstrated to inhibit Kv1.2, Kv1.5 and Kv4.3 channels. We determined the effect of this compound in 3 and 30 μ M concentration. Also, we applied ruthenium red (RR) as well and RR in 10 μ M concentration on Kv1.3 channels expressed in HEK-tsA 201 cells. Both of them are efficient blockers of Kv1.3 channels (estimated K_d assuming a Hill coefficient of 1 are 3 μ M and 4 μ M for AEA and RR, respectively). In addition the application of AEA also sped up the inactivation kinetics of the Kv1.3 current. To minimize the contribution of any endogenous channels we over-expressed Kv1.3 channels in HEK-tsA 201 cells and recorded current traces in outside-out patch configuration. These results clearly demonstrate that both molecules inhibit Kv1.3 channels with comparable potency to TASK channels. Hence, they are not suitable to separate Kv1.3 and TASK currents in lymphocytes.

4.1.5 Gating of Kv1.3 channels modified in T cells conjugated to APC

To study the possible functional consequences of this rearrangement on the Kv1.3 channels we determined biophysical parameters of whole-cell Kv1.3 current in D10 cells conjugated to B cells.

The activation of Kv1.3 channels in an APC-conjugated and in a standalone T cell (current traces normalized to the peak current were used to emphasize changes in the kinetics of the current). As for control cells, the activation of Kv1.3 current was also fitted in accordance with the Hodgkin–Huxley model. The interaction of T cells with B cells slowed the activation kinetics, which proved to be statistically significant ($\tau_{a,c} = 1.36 \pm 0.06$ ms, $\tau_{a,c} = 2.31 \pm 0.13$ ms ($n = 7$), $p < 0.001$). On the contrary, the inactivation rate of Kv1.3 current increases when T cell conjugates to a B cell. The statistical comparison of the inactivation time constants showed significant differences between control and IS-bound T cells ($\tau_{in,c} = 365 \pm 27$ ms, $\tau_{in,IS} = 263 \pm 29$ ms ($n = 7$), $p = 0.037$).

These changes in the kinetic parameters of activation and inactivation may influence the steady-state parameters of the activation. The midpoint of voltage-dependence of steady-state activation for T cells in IS ($V_{1/2,IS}$) was shifted toward positive potentials: -20.9 ± 2 mV ($n = 7$) with respect to the control cells ($V_{1/2,c} = -26.4 \pm 1.5$ mV ($n = 12$), $p < 0.05$). The slope factor (k) for IS-bound T cells was higher but statistical comparison did not show significant change with respect to the control ($k_c = 7.6 \pm 0.5$ mV, $k_{IS} = 9.3 \pm 0.8$ mV, $p = 0.115$). These modifications in the gating of Kv1.3 channels may indicate the phosphorylation or dephosphorylation of the channel protein upon diffusion into the IS, and rearrangement into distinct membrane domains.

4.1.6 PK antagonists speed up inactivation of Kv1.3 current

To clarify the role of protein kinases in the regulation of Kv1.3 channels segregated into the IS we applied H89, GF109203X (GF) and damnacanthal

(DAM), inhibitors of PKA ($K_d \approx 50$ nM), PKC ($K_d \approx 14$ nM) and p56^{lck} ($K_d = 17$ and 620 nM), respectively. It was reported previously that these compounds have direct blocking effect on ion channels: H89 blocks Kv1.3 with an IC_{50} of 1.7 μ M and GF inhibits Kv1.5 current with a K_d value between 1 and 2 μ M. Hence, the concentrations used in the experiments were set to avoid or reduce direct effect of the PK antagonists although they could still efficiently inactivate protein kinases (H89: 0.1 μ M, GF: 0.5 μ M, DAM: 1 μ M).

30-ms-long +50 mV pulses were applied in a control and three, PK antagonist treated cells. The activation kinetics of treated cells was also fitted according to the HH n^4 -model. The activation kinetics was not altered by PK inhibitors ($\tau_{a,c} = 1.36 \pm 0.06$ ms ($n = 18$); $\tau_{a,GF} = 1.51 \pm 0.17$ ms ($n = 9$), $p = 0.3$; $\tau_{a,H89} = 1.56 \pm 0.2$ ms ($n = 13$), $p = 0.3$, $\tau_{a,DAM} = 1.16 \pm 0.19$ ms ($n = 6$), $p = 0.22$).

When standalone D10 cells were pre-incubated with PK inhibitors the inactivation kinetics speeded up. Statistical comparison of control group versus treated groups proved that decrease in the inactivation time constant was significant ($\tau_{in,GF} = 252 \pm 32$ ms ($n = 9$); $\tau_{in,H89} = 254 \pm 20$ ms ($n = 12$); $\tau_{in,DAM} = 269 \pm 27$ ms ($n = 7$) $p < 0.05$).

Additionally, we also studied the effect of PK antagonist treatment on the voltage-dependence of the steady-state activation. The application of the PK inhibitors did not affect the equilibrium parameters of activation significantly ($V_{1/2,GF} = -27.6 \pm 1.3$ mV ($n = 7$), $V_{1/2,H89} = -24.8 \pm 2.4$ mV ($n = 10$), $V_{1/2,DAM} = -27.0 \pm 2.1$ mV ($n = 5$); $k_{GF} = 6.6 \pm 0.6$ mV, $k_{H89} = 7.7 \pm 0.6$ mV, $k_{H89} = 8.6 \pm 1.2$ mV, $p = 0.05$).

We also calculated the cell density (CD) values for each treatment: H89 and DAM treatment significantly reduced channel activity ($CD_{H89} = 89.4 \pm 14$ pA/pF, $CD_{DAM} = 85.9 \pm 20$ pA/pF, $p < 0.05$), whereas incubation with GF had no effect ($CD_{GF} = 163.1 \pm 25$ pA/pF).

4.2 Potassium channel expression in human CD4⁺ regulatory and naïve T cells from healthy subjects and multiple sclerosis patients

4.2.1 Characterization of human peripheral lymphocytes

We used short depolarizing pulses to measure the peak current of the voltage-gated K⁺ current component. During longer depolarizing pulses the whole-cell currents inactivated with kinetics that could be characterized by the time constant of the single exponential course of the decaying current. In the case of voltage-gated channels, the parameters describing the voltage-dependence of steady-state activation (G–V function) are characteristic for a given channel and thus can be used to identify the relevant channel and detect changes in its gating associated with certain physiological conditions. This was accomplished by recording current–voltage (I–V) relationships by measuring the current at increasing test potentials from –70 to +40 mV in 10 mV increments.

From these data the normalized conductance at each test potential was calculated to obtain the G–V function. The points were fitted with a Boltzmann-function and the voltage of half-activation ($V_{1/2}$) and the slope factor (k) were determined. The biophysical characteristics of the currents both in naïve and Treg cells correspond to the properties of Kv1.3 K⁺ channels, described previously as the predominant K⁺ channel in the membrane of T cells. The expression of other voltage-gated channels was not observed in either cell population. The measured peak currents indicated the presence of a few hundred channels per cell in agreement with previous results.

Besides Kv1.3 channels T cells also express the Ca²⁺-activated IKCa1 channel at levels depending on their activation state and subtype. Current through these channels is only activated at elevated Ca²⁺ concentrations so we set the [Ca²⁺] in the pipette solution to 1 μ M for these measurements. Due to their voltage-independent nature IKCa1 currents are most easily measured using voltage-ramps. During voltage-ramps the membrane potential is changed in a linear fashion, in our case from –120 to +50 mV, and the current changes

accordingly below the activation threshold of Kv1.3 channels. A steeper slope represents more IKCa1 channels, but the determination of the K^+ -specific current component also requires the measurement of the reversal potential.

In most cells we applied Charybdotoxin that blocks both channel types the extracellular solution which blocked the majority of both channel types. The remaining current corresponded to the passive leak current, which was then subtracted from the control current to get the IKCa1 component. Based on the current amplitude at a given voltage and the known single channel conductance of IKCa1 channels (11 pS) we could estimate the number of the channels in the membrane. We found no significant differences in the expression level of IKCa1 channels between Tregs and naïve cells from either donor type (healthy naïve: 12.4 ± 2.4 , healthy Treg: 21.2 ± 8.2 , MS naïve: 8.7 ± 2.1 , MS Treg: 22.9 ± 8.5 channels/cell).

With the voltage ramps we have not detected any current that could be due to ion channels other than the two K^+ channels in any of the cell types either.

Also, we examined the steady-state and kinetic parameters of the gating of Kv1.3 channels in the four different subpopulations. The analysis did not reveal differences between Tregs and naïve cells either from healthy donors or from MS patients.

4.2.2 Investigation of ion channel expression pattern on regulatory and naïve T cells from patients of multiple sclerosis and healthy objects

As great differences can exist in the expression level of Kv1.3 channels among different T cell subsets, first we compared the Kv1.3 current amplitudes. Since compromised functioning of Tregs was reported in MS patients, our strategy in statistical analysis was to compare the measured parameters between Tregs and naïve cells from healthy donors and examine if the same relationship exists between Tregs and naïve cells of MS patients. We found no difference in

the number of Kv1.3 channels expressed by naïve and regulatory T cells from healthy donors ($p = 0.125$, MWRST), but Tregs had a reduced number of channels compared to naïve cells in MS patients ($p = 0.005$, MWRST).

When T cells are activated they increase in size, which can be detected as an increase of membrane capacitance due to the larger surface area. Since at least a fraction of Tregs can be considered as being in a preactivated state we made a comparison of the measured capacitance values. Tregs from healthy donors had a significantly higher capacitance than their naïve counterparts ($p = 0.009$), but this difference was not seen between Tregs and naïve cells in MS patients ($p = 0.392$).

The current normalized to membrane capacitance (current density) provides information about the impact of the channels on the membrane potential control of the cell, since a given number of channels control the membrane potential more readily in a smaller cell than in a larger one. The current density (pA/pF) was calculated for each cell individually, that is, the peak current was divided by the membrane capacitance for each cell and these values were averaged for the group. When comparing the current densities we found a significant difference between naïve and regulatory cells in the healthy population ($p = 0.003$, MWRST), but not among the cells of MS patients. This lack of significant difference is the result of the significantly lower channel number of Tregs compared to naïve cells in MS patients accompanied by a slightly (but not statistically significantly) lower membrane capacitance.

5 DISCUSSION

During our electrophysiological measurements we characterized the whole-cell K^+ currents of D10 cells biophysically and pharmacologically and pointed out that this T_H2 cell line dominantly expresses the voltage-gated Kv1.3 channel.

Previous studies on various murine T cell lines showed that they express multiple K^+ channels, which were originally thought to produce type “n” and “l” currents. The former corresponds to the current of Kv1.3 channels; the latter is that of the Kv3.1 channels. They are both depolarization activated but basic biophysical (equilibrium parameters of activation and inactivation) as well as the pharmacological properties are distinct. The dose–response curves determined for TEA and MgTx and K_d values (13.4 mM for TEA and 16.8 pM for MgTx) showed that the Kv1.3 is the dominant voltage-gated potassium channel in D10 cell line. Of course, we should not exclude the possibility of heteromerization of these channels but the remaining current percentage (approx. $5 \pm 3\%$) at 500 pM of MgTx (approx. 30 times higher than the half-blocking concentration) or TEA dose–response clearly demonstrates that contribution of heteromeric constructs to the whole-cell current was negligible or zero.

Since we wanted to exclude the presence of the two-pore TASK channels as well, we tested the effect of 100 pM Margatoxin using a voltage-ramp protocol. Margatoxin is a well known Kv1.3 channel blocker; it blocks more than 85% of the Kv1.3 whole-cell current, however, TASK-1 and TASK-3 channels transfected into HEK-tsA cells did not show sensitivity. Meuth et al. used two compounds anandamide (AEA) and ruthenium red (RR) to separate Kv1.3 and TASK currents in lymphocytes. The change in the kinetics of the whole-cell current was also observed upon the administration of AEA. This may lead to the conclusion that selective removal of the TASK conductance by AEA alters the kinetics of the whole-cell current in T cells. So far no study has

reported whether these molecules would affect the current of Kv1.3 channels, moreover, ruthenium red was not tested at all for voltage-gated K^+ channels. Therefore we investigated the effect of specific blockers of TASK channels on Kv1.3 channels transfected into HEK-tsA cells. Anandamide in 3 μ M and ruthenium red in 10 μ M concentration significantly decreased the Kv1.3 current amplitudes (the estimated K_d values for Kv1.3 channels are similar to published data for TASK channels). Thus, these channel blockers are not specific, TASK currents cannot be distinguished clearly in lymphocytes.

Furthermore, since TASK channels are time- and voltage-independent, using voltage-ramp protocol on D10 cells. In order to eliminate the voltage-gated Kv1.3 channels, we applied 100 pM Margatoxin as a pharmacological block and we applied 0 mV -where Kv1.3 channels inactivate- holding potential as a biophysical block. Reversal potential was determined for -4 mV, which is much more positive as it would be in the presence of TASK channels. (theoretical value knowing the compositions of the solutions is – 85 mV). Based on these data we concluded that these channels are not expressed by the D10 cell line.

In the present study we demonstrated that redistribution of the predominant voltage-gated K^+ channels, Kv1.3 of a T_H2 cell line, D10 upon engagement to an APC affected the inactivation and activation gating of the ion channel. We investigated both equilibrium and kinetic parameters of activation and inactivation of Kv1.3 channels upon redistribution into the IS and found that it resulted in the following changes of channel operation: (1) decrease of the inactivation time constant, (2) slower activation kinetics; (3) the midpoint of steady-state activation but not the slope factor was altered. With the help of PK inhibitors we could demonstrate that the faster inactivation kinetics may be due to the extent of phosphorylation of the channel protein.

It was shown earlier that various cytosolic and membrane proteins – including Kv1.3 channel – accumulate in the IS formed between a T cell and an

APC. Here our data present that rearrangement of Kv1.3 into the IS between a D10 and a CH12 cell has functional consequences on Kv1.3 current, which might be attributed to the modification of tyrosine/serine/threonine amino acid side chains by protein kinases or phosphatases. Studies on PKC as well as PKA activation in human peripheral T cells demonstrated the increase in the whole-cell conductance of Kv1.3 current. Furthermore, upregulation of PKC function led to the decrease in the inactivation rate of Kv1.3 in T cells, and PKA activation resulted in slower inactivation of Kv1.5 channels in HEK-tsA cells also expressing Kv β 1.3 accessory subunit. The activation of the PKC altered the voltage-dependence of steady-state activation and inactivation, while PKA only affected the steady-state inactivation in such a manner that the window-current increased significantly (window-current gives the probability of the opening of ion channels around the resting membrane potential). Contrary, in a Jurkat T cell line or in HEK-tsA cells heterogeneously expressing Kv1.3 full or partial inhibition of the Kv1.3 current upon PKA/C stimulation was described. Generally, the activation of receptor and non-receptor tyrosine kinases was shown to reduce the magnitude of Kv1.3 current, although Fas-receptor activation led to the increase in Kv1.3 activity. These discrepancies could be attributed to the different expression pattern of PK isoforms or absence of specific linking proteins in different cell lines. Taken together, we suppose that experiments performed with PKA and p56^{lck} antagonist demonstrate the dephosphorylation of Kv1.3 channels upon recruitment into the IS. This scenario is verified by the faster inactivation kinetics of D10 cells to be in IS and the lower current density. Thus, we suppose that diffusion of Kv1.3 channel into the IS is a prerequisite for dephosphorylation of the channel. The molecular mechanism of channel modification in IS-engaged T cells still remains unclear, although we suspect that some protein phosphatases could account for the changes in Kv1.3 current described here.

The slower activation kinetics along with the shift of steady-state activation toward depolarizing voltages predicts the change in the microenvironment of Kv1.3 protein upon segregation into the IS. This assumption is also underlined by experiments with PK inhibitors: they only affected inactivation rate but neither kinetic nor equilibrium parameters of activation. A recent work from our laboratory described the similar phenomenon for Kv1.3 channels in T cells: the increase in the membrane viscosity, which was achieved by elevation of membrane cholesterol level, reduced the activation rate and shifted the voltage-dependence of the steady-state activation.

We reported that redistribution of Kv1.3 channels into the IS in APC-engaged T cells modified the parameters of the channel gating. Several PK inhibitors used in this study could not entirely explain the role of phosphorylation in the channel modification; hence we suppose that Kv1.3 channels may also diffuse into membrane domains with higher stiffness.

Regulatory T cells represent a small group within the large population of T cells that are emerging as very promising therapeutic targets due to their critical roles in controlling immune responses against self and non-self. As much as is known about the ion channels of T cells in general, up to now no study has examined this aspect of Tregs specifically or addressed the channel repertoire of Tregs in MS patients. With our study we filled this gap with the hope of detecting differences in ion channel expression that may provide a tool to selectively manipulate this important subset of T cells.

Overall, our results indicate that the numbers of Kv1.3 and IKCa1 channels expressed by all four groups of cells are similar to the numbers published earlier for quiescent T cells. However, statistical analysis reveals that Tregs of MS patients express fewer Kv1.3 channels than naïve cells unlike cells from healthy donors where such difference does not exist. If cell size, which is a determinant of channel density, is taken into consideration further differences

become apparent. Mitogenic stimulation of a mixed T cell population leads to a general increase in the size of the cells accompanied by an increase in the expression of Kv1.3 and IKCa1 channels. Our cells were unstimulated, yet the results show that Tregs have a significantly larger membrane surface area than naïve cells in healthy subjects without a difference in the number of ion channels expressed, which results in a significantly higher Kv1.3 channel density (pA/pF) of naïve cells than Tregs. These are in agreement with the notion that Tregs are in a partially or inadequately activated state and are anergic in response to antigenic stimulation. The increased surface area may be an indicator of the launching of certain pathways of activation, but the lack of the usual rise in the number of expressed K^+ channels may contribute to the cessation of the activation program that would lead to a “normally” activated T cell carrying on to proliferation.

We have found that the number of neither Kv1.3 nor IKCa1 channels was different between healthy Tregs and naïve cells. As the critical role of K^+ channels in T cell activation is the stabilization of the membrane potential during Ca^{2+} influx, the increase in cell size without the accompanying upregulation of K^+ channels may lead to poor membrane potential control resulting in an insufficient Ca^{2+} signal and ultimately a reduced tendency to proliferate. In contrast to cells from healthy donors, Tregs from MS patients expressed fewer Kv1.3 channels than naïve cells and did not have a larger membrane surface than naïve cells. The lower channel number and smaller size of MS Tregs suggests that some signaling pathways involving protein synthesis may be adversely affected by the disease interfering with regulatory T cell differentiation. This observation may be related to the reported reduction of the suppressive ability of Tregs in MS patients, but the connection must be further investigated.

Despite the lack of major qualitative differences in expression levels we did find differences in channel numbers, cell size and channel density, which are

likely to be associated with the activation status of the cells or their ability to be activated by mitogenic stimuli. The changes in the ion channel expression and cell size of isolated Treg populations upon activation would be worth investigating to better understand the nature of their activated state and probe the possibility of their pharmacological manipulation via ion channels.

6 SUMMARY

We carried out biophysical and pharmacological characterization of whole-cell K^+ current in D10 cells by patch-clamp technique in voltage-clamp mode. Excluding the presence of the two-pore TASK channels, we pointed out that this T_H2 murine cell line predominantly expresses Kv1.3 channels.

Using the CH12-LX cell line as antigen presenting cells we set a model system for studying the immunological synapse. We determined the cell surface distribution of Kv1.3 channels after the formation of the immunological synapse by labeling endogenous Kv1.3 channels against their extracellular epitope indirectly. Our confocal microscopy images demonstrated that Kv1.3 channels of these T cells accumulate in the contact region of the T cell – APC conjugate.

We compared the Kv1.3 currents, kinetic and steady-state parameters measured in standalone and APC-engaged cells. We found that activation kinetics slowed down, while inactivation rate increased in cells participating in the immune synapse, and we did not find significant difference in voltage-dependence of steady-state activation between standalone and T cells in the immunological synapse by plotting the normalized conductance as a function of test potential.

To clarify its background we applied PKA, PKC and $p56^{lck}$ inhibitors. The activation kinetics and the voltage-dependence of steady-state activation were not altered by PK inhibitors, however, our statistical comparison proved that the decrease in inactivation time constant was significant in treated cells compared to the control group. We suppose this phenomenon to be associated with the dephosphorylation of the channel protein in the immunological synapse.

Furthermore, we investigated the pattern of ion channel expression of naïve and regulatory T cells isolated from healthy volunteers and from patients with multiple sclerosis. We measured the numbers of the voltage-gated Kv1.3 and the Ca^{2+} -activated IKCa1 channels, studied the gating of these channels,

then we performed a statistical analysis among the different populations. Although, we did not find significant difference in gating parameters of Kv1.3 and the number of IKCa1 channels, we determined that the naïve and regulatory T cells of healthy donors express the same amount of Kv1.3 channels, and that the membrane capacitance is higher of regulatory T cells, thus the channel density is lower (current / capacitance), while the Treg population from MS patients expresses fewer Kv1.3 channels and their surface is not significantly larger compared to their naïve counterparts.

KEYWORDS

Immunological synapse, Kv1.3 channels, TASK channels, phosphorylation, protein kinase, regulatory T cell, autoimmunity, cell capacitance, channel density

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1. Hajdú, P., Szilágyi, O., **Tóth, Á.**, Krasznai, Z., Pocsai, K., Panyi, G.: Answer to the "comment on functional consequences of Kv1.3 ion channel rearrangement into the immunological synapse" by Stefan Bittner et al. [Immunol. Lett. 125 (Aug 15 (2)) (2009) 156-157].
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