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Study of T cell cation channels

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1. Introduction

An important step in the adaptive immune response is the activation of T cells, in which the physical interaction between the T cell and the antigen presenting cell (APC) results in polarization of the T cells. This leads to the accumulation of cell surface proteins and signaling molecules at the T cell-APC contact site, forming a highly organized signaling platform known as the immunological synapse (IS). The encounter with APCs, antigen presentation through the MHC molecule of APC activates the T cell TCR/CD3 complex, which initiates various signaling cascades and results in T cell activation. This leads to the activation of PLC (phospholipase C) and the formation of IP3 (inositol 1,4,5-trisphosphate). IP3 binds to the IP3 receptor in the membrane of the endoplasmic reticulum (ER), releasing Ca^{2+} from the ER. The CRAC channel is composed of the pore-forming Orai1 and ER-resident STIM1 subunits. The depletion of endoplasmic reticulum Ca^{2+} stores is sensed by STIM1, which undergoes a conformational change, multimerizes and translocates to the plasma membrane, where it binds to Orai1 in the plasma membrane, which activates Ca^{2+} influx from the extracellular space. This calcium influx requires an interplay between CRAC channels and voltage-gated Kv1.3 and Ca^{2+} activated KCa3.1 potassium channels. Potassium channels stabilize membrane potential between -40 and -50 mV, thus providing the driving force for Ca^{2+} influx through CRAC channels, which is essential for Ca^{2+}-mediated signaling and T cell proliferation.

It has been previously reported that increased expression of these ion channels (Kv1.3, KCa3.1, CRAC) is observed in activated human T cells and plays a role in Ca^{2+} signaling. There is clear evidence for the role of potassium channels that inhibition of ion channel function prevents the formation of Ca^{2+} signaling and cell division. For CRAC channels, a non-conductive mutation in the Orai1 subunit leads to complete loss of T cell function.

1.1 Characteristics of cation ion channels in T cells

Ion channels are pore-forming proteins found in almost all cell types and allow the rapid passage of various ions across the membrane (including the membrane of plasma and intracellular organelles) toward the electrochemical gradient. Several ion channels (e.g., most Na^+, K^+, Ca^{2+} channels) activated by changes in the voltage, but certain K^+ and Cl^- channels, CRAC channels, TRP channels, ryanodine receptors, and IP3 receptors (which are relatively voltage-insensitive) are regulated by secondary messengers and other intracellular and and / or extracellular mediators. In our work, we investigated the voltage-dependent Kv1.3, the Orai1 subunit of Ca^{2+}-activated CRAC channel, so we discuss them in detail below.

1.1.1 Kv1.3 channels

Potassium channels are essential players in setting the membrane potential and in the regulation of intracellular signaling in different cells. Voltage-gated potassium channels of the large family of K^+ channels (Kv channels) are comprised of four subunits (both hetero- and homomers) in native cells and heterologous expression systems. A Kv channel subunit consists of six α-helical transmembrane segments (S1-S6). The intracellular N-terminal region of the channel contains the tetramerization T1 domain, which is required for assembly of individual subunits in the ER. Furthermore, accessory Kvβ subunits can bind to the N terminus, and enable the binding of several signaling molecules, such as kinases. The highly-conserved pore region of Kv channels is formed by the linker between the S5 and S6, and mainly functions as a selectivity filter for K^+ ions. The fourth transmembrane segment, which contains several positively charged amino acid residues, is considered to be the voltage sensor of all Kv channels. The C-terminus of the channel can be coupled to various linker/adaptor proteins, which can anchor the protein to the cytoskeleton, bind to kinases or even regulate steering of the channels to the plasma membrane.

1.1.2 Orai1 and STIM1

In T cell activation, the influx of Ca^{2+} ions through CRAC channels is an important step. Depletion of ER Ca^{2+} stores is sensed by the STIM1 subunit located in the membrane of the endoplasmic reticulum. STIM1 then undergoes conformational changes, multimerizes, and translocates to the plasma membrane (PM), where it activates the PM-resident, Orai1 pore-forming domain. Orai1 has two isoforms: the longer is Orai1α, which consists of 301 amino acids (aa), and the shorter is Orai1β, which lacks the first 70 amino acids of the α isoform. The Orai1α channel, considered the wild-type variant, consists of six subunits. Each subunit contains four alpha-helical transmembrane domains (TMs) and has intracellular N- and C-termini and a selectivity filter at position 106. The interaction of the CRAC activation domain (CAD) of STIM1 and the CAD binding domains at the N- (73-91aa, α isoform numbering) and C-terminal end (342-448aa) of Orai1 results in channel opening.

STIM1 is a type I transmembrane protein and consist of 685 amino acid located in the ER at resting state. Depletion of the ER Ca^{2+} store results in a rearrangement of STIM1 from the ER close to the plasma membrane. The cytosolic C-terminal is essential for the delivery of STIM1 oligomers to ER-PM nodes and the subsequent opening of the CRAC channel, where

the CAD domains are linked to the CAD binding domains at the N- and C-termini of Orai1, thereby activating the channel.

Mutations in Orai1 and STIM1 may inhibit the function of the CRAC channel (eg, decrease in conductance, and thus the development of the immune response). Modification of the CRAC channel function or complete loss of function may result in immunodeficiency, infections, T-cell activation defects, congenital respiratory failure. Therefore, research on different phenotypes / mutants of Orai1 is extremely important to understand the mechanism of CRAC-associated diseases.

1.2 Membrane expression of Kv1 ion channels

Several studies have been published on the birth, membrane trafficking/targeting and assembly of channels. Though many membrane proteins have a cleavable signaling sequence for targeting to the plasma membrane, Kv1 channels lack this motif and the S2 segment serves as a recognition site for targeting. Other protein motifs were described in Kv1 channels that facilitate retention in the ER or forward targeting. For Kv1.4 channels the "VXXSL" motif of the intracellular C-terminus promotes high surface expression 11. The pore region of Kv1.4 channels also governs targeting to the membrane. However, the Kv1.1 channel lacks the "VXXSL" sequence, instead it possesses the "HRET" amino-acid motif right after the S6 segment in the C-tail. Introduction of a stop codon after the R or H residues of this latter sequence leads to a loss of K+ conduction without altering the cell surface expression level. It has been published that Shaker K+ channels, a Kv1 analogue in Drosophila, are also targeted to the plasma membrane without the "HRE" region of the C-terminal. The lack of the "HRE" region in Shaker resulted in a drastic change in the steady-state gating parameters, as opposed to the loss of the conductance as in Kv1.1. On the contrary, deletion of amino acids preceding the "HRET" sequence in Shaker, (still part of the C-terminal region) prevented channel expression. Kv1.3 was shown to be guided to the cell membrane by the presence of two acidic glutamate (E) residues at the C-terminus of the channel: removal of these amino acids with C-terminal deletion or mutation to isoleucine resulted in the loss of ionic current and plasma membrane expression.

1.3 Immunological synapse and T cell activation

In vivo activation of T lymphocytes occurs upon contact with antigen presenting cells (APCs). The physical interaction between T cell and APC results in the initiation of different signaling cascades leading to T cell polarization: the accumulation of cell surface proteins,

intracellular organelles, and signaling molecules in the immunological synapse (IS) between T cell and APC. Movement of specific cell surface proteins into IS is required for proper T cell activation. After the formation and presence of IS, several membranes (CD43, CD44, CD45) and intracellular proteins (p56Lck, SAP97, PSD-95, HS1, etc.)) are arranged, and increased polymerization of F-actin is observed in the T- in a cell.

1.4 Ion channels in the immunological synapse

Our group has previously shown that Kv1.3 ion channels are enriched in the immune synapse formed between T cells and APCs, and their function is altered by phosphorylation and dephosphorylation, respectively. Furthermore, PSD-95 protein, which is also present in T cells, has been shown to play an important role in the synaptic redistribution of Kv1.3 channel, and cortactin, which is an actin binding protein mediates the anchoring of Kv1.3 channel to the actin cytoskeleton during F-actin polymerization. Several studies have addressed the possible causes of SLE (systemic lupus erythematosus) disease, where it has been found, that the dynamics of the arrangement of Kv1.3 in IS in SLE T cells differs from that of healthy T cells, which has been shown to contribute to the development of higher cytosolic Ca^{2+} levels in the T cell, which may cause hyperactivity of SLE T cells. Furthermore, for Kv1.3, inhibition of channel diffusion into IS by antibody cross-linking has been reported to result in a modification of Ca^{2+} signaling at the immunological synapse.

In addition, both subunits of the Ca^{2+} activated CRAC channel (Orai1 and STIM1) have been reported to accumulate in the IS. It has been shown that Orai1 is able to localize to the synapse in both Jurkat and human T cells, even when the non-conducting mutant of Orai1 (E106) is expressed in the cells. Furthermore, the influx of calcium through CRAC channels promotes the polymerization and arrangement of actin filaments in the immunological synapse, leading to rearrangement of the ER and thus diffusion of Orai1 and STIM1 into IS.

Based on this, it can be hypothesized that immobilization of ion channels of T cells (Kv1.3, KCa3.1, Orai1, STIM1) may influence the outcome of T cell activation, and the residence time of ion channels in IS may be important for the Ca^{2+} response required for physiological development.

1.5 MAGUK proteins, SAP97

DLG1, also known as synapse-associated protein 97 (SAP97), is a scaffold protein belonging to the MAGUK family of proteins. It is widely expressed, especially in the epithelial cells of the body. Like other MAGUK proteins, SAP97 consists of three PDZ domains, a single

SH3 domain and a GUK domain. SAP97 has been shown to interact with a number of partners through protein-protein interaction domains, including membrane ion channels and cytosolic proteins. SAP97 is able to bind to potassium and sodium ion channels in cardiac cells, thereby regulating cardiac action potential and rectifier currents, and SAP97 plays an important role in controlling the localization and anchoring of potassium channels in neurons. Furthermore, it has been reported that SAP97, together with other adapter proteins (PSD-95), accumulates in the immunological synapse and after 15 min it shows reduced accumulation between T cells and CD3-CD28 antibody-coated beads.

2. Objectives

I. The membrane expression and conductivity of Kv1 channels can be regulated by different protein motifs (VXXSL, HRET), which are mainly located at the C-terminus of the channel protein. The voltage-gated Kv1.3 channel plays an important role in the regulation of the Ca^{2+} signal during T cell activation. Many adapter proteins are able to bind to its C-terminus, as well as the "HRET" motif, so it is important to understand how the function and expression of the channel is altered in their absence (C-terminal, HRET sequence). Therefore, in the case of the Kv1.3 channel, we aimed to answer the following questions:

1. ***How does the partial or complete removal of the C-terminus of the Kv1.3 channel affect the cell surface expression and /or conductivity of the channel?***
2. ***What is the role of the HRET(E) sequence for the Kv1.3 channel?***
3. ***Can the HRET(E) sequence be replaced by other amino acids?***
4. ***What are the consequences of this substitution on the expression and/or function of the channel?***

II. It has been previously reported that Orai1 channels play an important role in Ca^{2+} signaling during T cell activation and are enriched in the immunological synapse between T cell and APC, but the kinetics of this redistribution is unknown. For other ion channels (e.g. Kv1.3) has been shown that binding of different adapter proteins affects the accumulation of channels in the synapse and their intracellular location. Based on these, we sought answers to the following questions in the case of Orai1:

1. What is the accumulation kinetics of the wild-type Orai1 channel in the immunological synapse formed between Jurkat T-cell and CD3-CD28 antibody-coated beads?

2. In the case of the N-terminally truncated Orai1, which is similar to the β isoform, does the dynamics of IS accumulation change?

3. How do different intracellular proteins that bind to Orai1 (STIM1, SAP97) affect synapse redistribution?

4. What are the functional consequences of different modifications (gene silencing, mutation) in single and synaptic T cells?

3. Materials and Methods

3.1 Cell Culture

Jurkat cells were cultured in RPMI solution supplemented with 10% FBS, 2 mM L-glutamine, 1mM Na-pyruvate, and 200 units penicillin/streptomycin.

HEK-293T, CHO cells were cultured in DMEM medium, which contained 10% FBS, 2mM L-glutamine, 1mM Na-pyruvate, and 200units penicillin/streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% of CO_2 and 95% air. Cells were passaged every 3 days.

In order to exclude the effects of the expression system on the Kv1.3 ion channel (eg the presence of endogenous Kv1.x channels / subunits that may promote the formation and expression of heteromeric channels in the membrane), the experiments presented in this thesis were performed in CHO cells, as they showed no measurable whole-cell current at + 50mV.

Transient transfection of DNA plasmids was performed using Lipofectamine 2000TM according to the manufacturer's protocol.

3.2 Plasmids and Cloning

The wild-type human Kv1.3 channel was subcloned into pEGFP-C1 plasmid using Bgl II and EcoR I restriction sites. For sticky-end ligation, T4 DNA ligase was used according to the standard protocol. The deletion (ΔC, NOHRET, HRETE) and insertion/point (FLAG, XHRETE, polyA, Atail) mutants were generated with flanking-primer mutagenesis method. Point-mutant (H399K, A413V), full-length constructs were expressed in pRc-CMV vector.

For co-transfection of wild type (WT) and mutant Kv1.3 plasmids mCherry tagged WT Kv1.3 was generated in two steps with PCR-based cloning: first EGFP was replaced with mCherry in pEGFP-C1 plasmid, then the wild-type Kv1.3 gene was subcloned into this pmCherry-C1 plasmid with EcoR I and Bgl II enzymes.

The wild type Orai1 channel (mGFP-Orai1-Full) obtained from Richard Lewis' lab and the N-terminally truncated Orai1 (mGFP-Orai1-Δ72) channel, in which the first 72 amino acids were deleted, were tagged with an mGFP at the N-terminal region and subcloned into the retroviral pBMN–LacZ vector using Hind III and Not I restriction sites.

Briefly, the PCR-amplified constructs were run on 1% agarose gel, then cut and purified with phenol extraction method. Afterwards, both the insert and the plasmid were digested with Hind III and Not I enzymes and cleaned up using ethanol precipitation methods. For sticky-end ligation, T4 DNA ligase was used according to the standard protocol. All constructs were sequenced at the Clinical Genomics Center at University of Debrecen.

3.3 Transformation

Plasmids prepared by PCR were added into 200 µl of E. coli competent cells and incubated on ice for 20 minutes. Heat shock (50-60 s) was applied at 42°C and the samples were again placed on ice. 800 µl of SOC medium was then added to the cells and shaken for 1 hour at 200 rpm at 37°C. Cells were plated on agar medium containing appropriate antibiotics (ampicillin or kanamycin). Cells in the petri dish were grown for 16 hours at 37°C.

3.4 Plasmid preparation

After transformation, bacterial colonies from the agar plates were inoculated into the appropriate antibiotic-containing LB or TB medium and shaken for 16 hours at 37°C, 200 rpm. PureYield Plasmid Miniprep System was used to purify small amounts of plasmid DNA and PureYield Plasmid Maxiprep System was used to purify larger amounts of plasmid.

3.5 Membrane Targeting

Different Jurkat cells expressing Orai1 channel were put into glass bottom chamber slides and were labelled with Alexa Fluor^{TM} 647 NHS ester in 1 × TBS for 20 min on ice. NIKON T*i*2 microscope was used to take confocal images of the cells and the ImageJ software was used for evaluation.

3.6 Gel electrophoresis and Western Blotting

Protein samples were separated by SDS–PAGE and transferred to PVDF membranes after electrophoresis. The membranes were blocked with milk powder and immunoblotted with mouse anti-STIM1, anti-FLAG M2,mouse anti-SAP97 and rabbit anti-actin primary antibodies. Then membranes were washed with TBS containing tween 20, and anti-mouse IgG HRP-linked or anti-rabbit IgG HRP-linked secondary antibodies were added to the membranes. After membranes were washed with TBS and distilled water, then blots were developed with ECL reagent and visualized with the FluorChem Q MultiImage III Western blot imaging system In each experiment 1×10^6 cells were harvested.

3.7 Coomassie-staining and GST affinity chromatography

For Coomassie staining, pGEX-4T-2-Orai1 N- or C-terminal GST fusion proteins and pGEX-4T-2-GST proteins (used as negative controls) were produced in Escherichia coli (BL21). Bacterial lysates were induced with 1 mM isopropyl beta-D-thiogalactoside (IPTG) for 2 hours at room temperature, then collected by centrifugation and washed three times with

1×PBS. Then it was sonicated in lysis buffer, run on an SDS gel and stained with Coomassie dye for 1 minute, then washed for 16 hours in a solution containing 12% ethanol and 7% acetic acid to check the protein production.

PGEX-4T-2-GST and GST-Orai1-1-72 bacterial lysates from previously harvested bacterial proteins were bound to GST SpinTrap columns for 1 hour during GST affinity chromatography. Jurkat cells (containing SAP97 proteins) were washed three times with 1xPBS, sonicated in lysis buffer and added to GST columns (with the pre-bound, appropriate GST construct) and incubated overnight at 4°C. The next day, the GST SpinTrap columns were washed and the proteins bound to the columns were eluted with 10 mM glutathione. The eluates were separated by SDS-PAGE and transferred to a PVDF membrane. The presence of proteins was confirmed by western blot using a special anti-SAP97 antibody.

3.8 Viral Transduction

Transduction protocol for Jurkat cells was obtained from the Nolan's Lab web page (http://www.stanford.edu/group/nolan/, accessed in 2010).

3.9 Knockdown of STIM1 and SAP97 with shRNA

STIM1 and SAP97 knockdown Jurkat cells were obtained using the retroviral transduction protocol with the help of Mission shRNA (mGFP-Orai1-Full-STIM1-KD and mGFP-Orai1-Full-SAP97-KD). For transduction/knock-down control, we used the pLKO.1-puro plasmid vector which contains an shRNA insert that does not target any known genes from any species (mGFP-Orai1-Full-PURO). The knock-down efficiency was assessed using western blot.

3.10 Immunfluorescence

CHO cells expressing FLAG bearing EGFP-Kv1.3 plasmids were plated onto poly-L-lysine coverslips and incubated for 3-4 hrs (37 °C, humidified, 5 % CO_2). Then cells were fixed with 1% formaldehyde and labeled with mouse anti-FLAG M2 antibody. Secondary antibodies (goat anti-mouse with Alexa647) were added to the cells for 1 hour. Finally, coverslips were mounted onto slides with Fluoromount G. Zeiss LSM 510 META and Olympus FV-1000 microscopes were used to take confocal images of the cells. A He-Ne laser was selected to excite Alexa647 and mCherry and an Argon laser to visualize EGFP. The thickness of the slices was set to 1µm.

3.11 Immunological Synapse Formation

IS was formed between Jurkat T cells stably expressing the Orai1 wild-type (mGFP-Orai1-Full), Orai1-N terminally truncated channels (mGFP-Orai1-Δ72), Orai1 wild-type missing STIM1 (mGFP-Orai1-Full-STIM1-KD), Orai1 wild-type missing SAP97 (mGFP-Orai1-Full-SAP97-KD), Orai1 wild-type transfected with control shRNA (mGFP-Orai1-Full-PURO) and CD3-CD28 beads and co-centrifugated at 200×*g* for 1 min at 37 °C.

Then cell-bead mixtures were plated on poly-L-lysine coated coverslips and were incubated for 1, 5, 15, 30 or 60 min (at 37 °C, and 5% of CO_2 and 95% air), and were placed on ice for fixing and fixed with acetone for 1 min. Between each step the cells were rinsed three times with 1 × TBS containing 1% BSA. F-actin accumulation at the IS was detected with Alexa-546 phalloidin, which was an indicator of IS formation. Finally, coverslips were mounted onto slides with Fluoromount G. Experiments with mGFP-Orai1-Full-PURO, mGFP-Orai1-Full-SAP97-KD and mGFP-Orai1-Full-STIM1-KD cells were performed in parallel.

3.12 Confocal Microscopy and Image Analysis

For Kv1.3 channel, images were recorded using Zeiss LSM 510 META and Olympus FV-1000 microscopes. He-Ne laser (633 nm laser line) was used to excite Alexa647, argon laser (488 nm line) was used to excite GFP, and the slice thickness was approximately 1μm.

NIKON T*i*2 microscope was used to take confocal images of the cells (the thickness of the slices was 1μm). For excitation of mGFP the line 488 nm of Ar-ion laser, for A546 the line of 561 nm of He–Ne laser was used, and emitted light was detected through 505–550-nm bandpass, 550–640 nm bandpass filters, respectively.

To determine the residency time of Orai1 channels in the IS, confocal images of Jurkat T cells engaged to CD3–CD28 beads were recorded at 1, 5, 15, 30, and 60 min time-points. The criterion of the IS formation was that T cell showed F-actin (A546-phalloidin) polarization at the bead-cell contact.

For the evaluation of confocal images, we used the ImageJ: same-sized squares were drawn as ROIs at the IS (bead-cell contact area, according to F-actin polarization using A546-phalloidin signal (6 squares)), outside the IS (including the membrane and intracellular region, 3 squares) and at a cell-free area for the background (3 squares), respectively. The accumulation ratio (AR) was defined by the following equation :

$$\mathrm{AR} = \frac{\text{Mean intensity of Orai1 in IS} - \text{Mean intensity of background}}{\text{Mean intensity of Orai1 outside the IS} - \text{Mean intensity of background}}$$

Cells were defined Orai1-polarized if AR was higher than 1.5.

3.13 Intracellular Ca2+ Measurements

First, the different Jurkat cells expressing Orai1 were plated onto poly-L-lysine coated glass bottom petri dishes. Then the cells were loaded with 1μM FURA-2 acetoxymethyl ester dissolved in DMSO and incubated for 25 min at 37 °C in phenol-red free RPMI solution. The cells were washed with 2 mM Ca^{2+} solution and were placed on a 37 °C stage of an inverted fluorescence microscope. The cells were perfused with 2 mM Ca^{2+} solution, 0 mM Ca^{2+} solution and then 1 μM Thapsigargin (TG) containing 0 mM Ca^{2+} solution was applied to deplete ER Ca^{2+} stores. After store depletion, the addition of extracellular 2 mM Ca^{2+} containing 1 μM TG activated intracellular Ca^{2+} elevation through SOCE.

Experiments with FURA-2 were completed with NIKON ECLIPSE T*s*2R microscope combined with a VisiChrome High Speed Polychromator. FURA-2 dual excitation and emission were accomplished using 340- and 380-nm excitation filters and a 510-nm emission filter. Images were recorded with a PCO Edge 4.2 sCMOS Camera at 10 s intervals and were analyzed with VisiView® Imagingg software. Only mGFP expressing cells were selected for the evaluation.

3.14 Intracellular Ca2+ Measurements in IS

Different Jurkat T cells stably expressing Orai1channels were plated onto poly-L-lysine coated glass bottom petri dishes and were loaded with 1 μM FURA-2 acetoxymethyl ester dissolved in DMSO and incubated for 25 min at 37 °C in phenol-red free RPMI solution. The cells were washed with phenol-red free RPMI solution and were placed on a 37 °C stage of an inverted fluorescence microscope and the Ca^{2+} signal was recorded as described above. CD3-CD28 beads was pipetted onto Jurkat cells (mimicking immunological synapse) and Ca^{2+} responses were measured until 90th minute after contacting with beads.

For evaluation, the Ca^{2+} signal in each cell was background corrected and the number of oscillations (peaks) in the 90-min period was determined. Those points were regarded as oscillatory peak that were higher than the average baseline (before adding the beads to cells) + 2 × SD (of the baseline), and height of the points before and after the peak was lower than the peak. Cells were measured on 3 different days. Only GFP expressing, “bead-activated” cells were selected for evaluation.

3.15 Electrophysiology

CHO cells transfected with various Kv1.3 channel constructs were washed with standard extracellular solution, and plated onto cell culture 35-mm petri dishes. Kv1.3 currents were recorded in whole-cell and outside-out configuration using an Axopatch 200B amplifier. The pipette filling solution consisted of 140mM KF. For pharmacological measurements a solution contanined TEA-Cl (tetraethylammonium). For gating-current and single-channel measurement the external bath did not contain Na^+, because Na^+ had to be omitted from the solution since some CHO cells expressed inward Na^+ current. P/5 protocol for online leak subtraction was applied to minimize capacitance and leak errors during the measurements with the A413V mutant. Perfusion of the cell with different test solutions was achieved using a gravity-flow perfusion setup, and excess fluid was removed continuously.

The current density was defined as the ratio of peak current detected at + 50 mV test potential and the whole-cell capacitance (read from the compensatory circuit of the amplifier). The remaining fraction of the current (RF) for TEA inhibition was defined as the ratio of the peak current measured after and before perfusion with 100 mM TEA.

The activation kinetics of the current was characterized by fitting the Hodgkin-Huxley (HH) model to the rising phase of the current traces evoked by 15-ms-long depolarizations to +50 mV. The activation time constant characteristic of a given cell was determined as the average of the time constants obtained upon three sequential depolarizations repeated every 15s.

The inactivation kinetics of the current was characterized by fitting a single exponential function to the decaying part of the current traces evoked by 2-s-long depolarizations to +40 mV from a holding potential of −120 mV. The inactivation time constant for a given cell was determined as for τ_a, except pulses were delivered every 60 s.

The voltage-dependence of steady-state activation relationships were obtained as follows. The cells were held at −120 mV holding potential and depolarized to various test potentials ranging from −70 up to +50 mV in 10 mV steps at every 30 s. Peak whole-cell conductance (G(V)) at each test potential was calculated from the peak current (I_p) at test potential V and the K^+ reversal potential (E_r=–85 mV) 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 and the Boltzmann-function was fitted to the data points: $G_N = 1/(1+\exp[-(V-V_{½})/k]$, where G_N is the normalized conductance, V is the test potential, $V_{½}$ is the midpoint and k is the slope of the function.

Gating currents were determined using a non-conducting Kv1.3 mutant (WT-W384F). A voltage step protocol was applied from -100 mV up to 20-100 mV with an increment of 10 mV, each step lasted for 50 ms, P/5 protocol was used for leak subtraction (pulses were opposite to the test potential) to reduce capacitance and leak errors during the measurements. The gating charge was calculated upon the integration of the area under gating current traces.

3.16 Statistical Analysis

Data are reported as the mean ± standard error. Means were compared using Student's t-test or one-way ANOVA. P-value was set to 0.05. Statistical analyses were performed using SigmaPlot version 10.0 (Systat Software Inc.).

To compare the percent of polarized cells we applied Fisher's exact test. To compare the AR values and number of Ca^{2+}-peaks for each sample we used one-way ANOVA on ranks or rank sum test. The level of significance was set to 0.05. For each time point and each cell line we collected data on $N \geq 3$ days, and number of cells per day was $n \geq 9$.

3.17 Flow cytometry

Analysis of cells were performed with Novocyte flow cytometer system and NovoExpress software, where at least 200,000 cells per sample were used. Immunostaining of different Jurkat cell lines (live cells, on 4 C) was carried out using Alexa Fluor 647 conjugated anti-human CD3 or CD28 antibody. Based on the dot plots created in NovoExpress software using statistics, the geometric mean and CV (coefficient of variation = SD/mean*100%) values are indicated for CD3 or CD28 in different cell lines. Only GFP-positive, live cells were included in the evaluation and unlabeled Jurkat cells were applied as control.

3.18 Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

4 Results

4.1 The role of HRET sequence in membrane expression and gating of Kv1.3

4.1.1 Strategic considerations for designing the Kv1.3 constructs

Three strategies were applied for unique identification of the Kv1.3 subunits transfected into HEK or CHO cells. First, we introduced the A413V mutation in the S6, which has been shown to accelerate dramatically the inactivation kinetics of A413V homomers as compared to wild-type channels (τ_{A413V} = 4 ms, τ_{WT}=200 ms).

Second, we substituted histidine (H) for lysine (K) at residue 399, which resulted in a TEA (tetraethylammonium) - insensitive, phenotype, as described earlier. The assembly of TEA sensitive (K_d ≈11.4 mM) and insensitive (K_d≈2000 mM) Kv1.3 subunits modifies the affinity of the heterotetramer for TEA, hence the heteromultimer formation can be easily identified by the application of 100 mM TEA.

Third, for immunocytochemistry experiments we used constructs that had the FLAG insert in the extracellular loop between the S1 and S2 segments. The insertion of the FLAG epitope does not alter dramatically the properties of Kv1.3 as shown earlier but allows labeling of the channels expressed in the plasma membrane.

For each construct, channel proteins were fused to the N-terminus with EGFP. Deletions at the C-terminus were generated using a stop codon inserted before the "HRET" sequence (construct labeled ΔC) or after the "HRET" sequence (labeled NOHRET).

4.1.2 Deletion of the C-terminal region does not influence membrane expression

Expression of the A413V-ΔC construct results in a robust whole-cell current having fast inactivation kinetics, as it was seen for full-length homotetrameric A413V. In CHO cells transfected under identical conditions with the A413V-NOHRET construct did not show any voltage- and time-dependent current.

Thereafter we tested whether all these channel constructs are properly translated by the cells using western-blot against a FLAG epitope inserted between the S1-S2 helical segments of all the constructs. CHO cells transfected with any of the three FLAG-tagged constructs express EGFP-tagged Kv1.3 channel subunit of appropriate size (between 85-95kDa).

4.1.3 Pharmacology reveals homomers of H399K-ΔC channels in the membrane

Measurements of the FLAG-H399K-FL mutant in CHO cells in control extracellular solution and in the presence of 100 mM TEA (inhibitor of K^+ channels, fast open-channel blocker) show

the overlapping currents indicate the lack of inhibition. Mutation of histidine (H) to lysine (K) resulted in a TEA-insensitive channel. When CHO cells were transfected with the FLAG-H399K-ΔC plasmid we measured currents similar to the full-length H399K construct. The C-terminal deletion that includes the "HRET" motif as well (FLAG-H399K-NOHRET) resulted in a phenotype that lacks outward K^+ current, just as we described for the A413V mutant .

4.1.4 NOHRET constructs also target to the cell membrane

FLAG-tagged full-length, H399K and A413V mutant Kv1.3 channels, were stained with anti-FLAG antibody in CHO cells and the staining shows a typical patchy distribution. 32. The same was observed for both Kv1.3 point mutants (A413V, H399K) that lacked either the ΔC or the C-terminus plus the "HRET" motif. Although in C-terminally truncated channels showed higher intracellular retention compared to full-length channels, the ΔC and NOHRET channels can be expressed in the membrane.

4.1.5. Co-expression of wild-type and A413V-NOHRET subunits results in pure wild-type Kv1.3 current

We co-transfected mCherry-tagged full-length, wild-type Kv1.3 channels along with EGFP-A413V-NOHRET plasmid mutants at 1:1 ratio. We found that a CHO cell expressing both A413V-NOHRET and WT-Kv1.3 exhibits currents resembling the pure WT current ; and not the "mixture" of multiple heteromeric currents characterized with various inactivation time constants. The current density of only WT-Kv1.3 channel expressing cells was far higher than those transfected with both WT-Kv1.3 and A413V-NOHRET constructs.

Moreover, we transfected CHO cells with a mixture of FLAG-tagged A413V-NOHRET and WT-Kv1.3 (mCherry conjugated) and subjected them to anti-FLAG labeling: microscopic confocal images clearly show the membrane expression of channels.

4.1.6 Gating charge movement of NOHRET channels is absent

To disclose if the conducting pathway or the activation gating is destroyed upon HRET removal in the NOHRET Kv1.3 we assessed the gating properties of WT-NOHRET construct expressed in CHO cells.

As a positive control, we expressed the WT-W384F channel, which is a non-conducting mutant of Kv1.3 (homologous to the non-conducting W434F mutant of the Shaker channel. Gating currents were recorded in a CHO cells stably expressing Kv1.3-W384F (we recorded gating currents in all 11 cells). When we measured the gating current in cells expressing WT-NOHRET channels no gating current was detected (n=9) or a miniature gating current was

detected at very depolarized test potentials of +50 mV or higher. These indicate that voltage-sensor movement of the channel is compromised when HRET is not present.

4.1.7 Deletion and substitution of the HRET(E) sequence does not affect the function of Kv1.3

Motivated by these findings, the following mutations were introduced:

1) HRETE motif was deleted, the remaining fraction of C-terminal was still present (WT-XHRETE);

2) HRET(E) was replaced with a run of five alanines (WT-polyA channel);

3) HRET(E) was replaced with a run of five alanines and the remaining part of C-terminal was deleted from the sequence (WT-Atail).

After transfecting these mutants and the WT full-length in CHOs we could show that HRETE-manipulated subunits formed functional and conducting tetramers in the CHO cell membrane, and no major differences in the kinetic and equilibrium parameters of the gating were found.

The activation kinetics was a bit slower for the WT-XHRETE and A-tail constructs, compared to WT-FL channel ($p<0.001$ for both), but inactivation kinetics were identical for every channel type ($p=0.13$). The half-activation voltage ($V_{½}$) of equilibrium activation was different only for WT-Atail channels, but the slope factor (s) were the same for all HRETE mutants.

Furthermore, we assessed the single-channel conductance for all four phenotypes and obtained that removal/replacement of HRETE-motif in Kv1.3 channels did not influence the unitary conductance ($p=0.085$).

In addition, all Kv1.3 channels carrying the FLAG epitope were detectable in the membrane upon labeling with FLAG antibody (these figures are not shown in the dissertation). All of these data clearly demonstrate that the HRETE motif is not vital for Kv1.3 function: the channel is present in the plasma membrane and functional even in the absence of the HRET(E) motif.

4.2 The presence of Orai1 in IS alters the Ca^{2+} repsonse of T cells

4.2.1 Kinetics of Orai1 redistribution into the immunological synapse

To study the kinetics of Orai1 channel accumulation in the IS, we established Orai1 stably expressing cell line in Jurkat E6-1 cells. First, we tagged Orai1 channel with mGFP, and using retroviral transduction mGFP-Orai1-Full construct was expressed in Jurkat cells. IS-formation

was initiated between anti-CD3 and anti-CD28 antibody coated beads and mGFP-Orai1-Full expressing cells, and the percentage of Orai1-polarized cells was determined at five subsequent time points (1, 5, 15, 30 and 60 min). To confirm the development of IS formation F-actin polymerization was monitored with the help of Alexa-546 phalloidin staining, and the accumulation ratio (AR) was introduced to decide if a T cell exhibited Orai1-polarization.

1 min after cell-bead encounter formation 20% of T cells showed Orai1-accumulation in the IS, and the portion of Orai1-polarized cells peaked at the 15th minute (46%). At later time points (30 and 60 min: p=0.033) the percent of cells exhibiting Orai1 IS-redistribution declined gradually. Next we studied how Orai1 is steered to the IS in Jurkat T cells.

4.2.2. STIM1 controls the IS-dwelling time of Orai1

To understand the function of STIM1-Orai1 interaction in Orai1 IS-redistribution, we established an mGFP-Orai1-Full cell line, in which we knocked down the STIM1 gene (mGFP-Orai1-Full-STIM1-KD). As a control for transduction the pLKO.1-puro plasmid was applied, which contains shRNA sequence that does not target any known genes (mGFP-Orai1-Full-PURO). To test the efficiency of STIM1 knock down, the STIM1 expression level was assessed using western blotting. Furthermore, we assessed the membrane targeting of mGFP-Orai1-Full subunits: Orai1 subunit showed membrane localization, the fluorescent signal of membrane staining and mGFP-Orai1-Full proteins are overlapping.

For functional checkup, Ca^{2+} signaling of mGFP-Orai1-Full-PURO and mGFP-Orai1-Full-STIM1-KD Jurkat cells were characterized by the measurements of the cytosolic Ca^{2+} changes using FURA-2 ratiometric imaging. The control cells (mGFP-Orai1-Full-PURO) had a typical Ca^{2+} response seen for mGFP-Orai1-Full and -as expected and published before- STIM1 knock-down completely abolished Ca^{2+}-response of cells through CRAC channels .

Afterwards, time-dependence of IS-accumulation for Orai1 channels in mGFP-Ora1-Full-PURO and mGFP-Ora1-Full-STIM1-KD cell lines was determined. The time course of the Orai1 IS-accumulation for mGFP-Orai1-Full-PURO cells was similar to mGFP-Orai1-Full construct expressing cells: percentage of Orai1-polarized cells peaked at 15 min (app. 40%) and dropped to ca. 25 percent just as seen for WT-Orai1. However, STIM1 knockdown modified the kinetics of Orai1 IS redistribution: the fraction of cells showing Orai1 accumulation in the IS increased up to 60th min in mGFP-Orai1-Full-STIM1-KD cells (p=0.029).

4.2.3 N-Terminal truncation of Orai1 mimics STIM1 knock-down

The C terminus of Orai1 subunit is essential for STIM1 coupling and SOCE activation, and N-terminal region contains a CAD binding domain that is necessary for the Ca^{2+} permeation. Therefore, we created an N-terminal mutant named mGFP-Orai1-Δ72 (mimicking Orai1β isoform and preserving N terminal binding of STIM1 partly), where the first 72 amino acids were removed to unveil the role of the intracellular amino-tail in the reorganization to the IS.

The truncation of the Orai1 (Orai1-Δ72) had no significant effect on the Ca^{2+} response of Jurkat cells, hence it did not influence significantly activation of the CRAC channel.

Intensity profile analysis shows that intensity peaks of Alex647 staining (membrane labeling) and the mGFP-signal overlap just as for the wild-type Orai1. Consequently, mGFP-Orai1-Δ72 channels are able to target the cell membrane.

Furthermore we formed IS between Jurkat T cells stably expressing N-terminally truncated Orai1 channels and CD3-CD28 antibody coated. Representative confocal snapshots demonstrate that mGFP-Orai1-Δ72 could redistribute into the IS but the percent of truncated Orai1-expressing mGFP-polarized cells gradually elevated and reached a peak at 60^{th} minute with 58% (p = 0.035 between mGFP-Orai1-Full and mGFP-Orai1-Δ72) similar to mGFP-Orai1-Full-STIM1-KD cells.

4.2.4 SAP97 could control the leaving of Orai1 from the immunological synapse

To unveil the role of the SAP97 in Orai1 trafficking to the IS we knocked down the SAP97 in Jurkat cells expressing mGFP-Orai1-Full channel (mGFP-Orai1-Full-SAP97-KD). As a control for transduction, the pLKO.1-puro plasmid was applied as previously (mGFP-Orai1-Full-PURO). The efficiency of SAP97 knock-down was tested using western blotting.

We checked the membrane expression where the intensity profile of Jurkat cells missing SAP97 and expressing the mGFP-Orai1-Full channels clearly demonstrates the membrane localization of the Orai1 subunit is not affected by the knockdown of SAP97.

To test the functionality of Orai1 channels in mGFP-Orai1-Full-SAP97-KD cells, the cytosolic Ca^{2+} changes were measured using the ratiometric Ca^{2+} indicator FURA-2. The transduction of the mGFP-Orai1-Full cells with SAP97 shRNA construct had no significant effect on CRAC current in Jurkat cells compared to the mGFP-Orai1-Full-PURO cells.

Thereafter, the kinetics of Orai1-accumulation was determined in SAP97 knocked-down cells, where the percent of Orai1-polarized cells at the 60th minute (p=0.029) was higher as compared to the control.

4.2.5 Sustained Orai1 IS-Residence Modifies Ca2+-Signaling

To test the functional consequence of sustained Orai1 IS-residency we measured the Ca^{2+} responses in Jurkat cells forming immunological synapse with CD3-CD28 antibody coated beads. Our results showed increased number of calcium peaks in mGFP-Orai1-Δ72 (compared to control mGFP-Orai1-Full, p=0.016) and mGFP-Orai1-Full-SAP97-KD (compared to control mGFP-Orai1-Full-PURO, $p < 0.001$) during the first 60-min period of CD3-CD28 bead engagement. After 60 min the calcium oscillations were still present in N-terminally truncated Orai1 and in Orai1-Full expressing SAP97-KD cells unlike in control cells: the number of oscillatory peaks were significantly lower in control cells (mGFP-Orai1-Δ72 vs. mGFP-Orai1-Full with $p< 0.001$; mGFP-Orai1-Full-SAP97-KD vs. mGFP-Orai1-Full-PURO with p=0.002, between the 60^{th} and the 90th minute).

In addition, the time course of Ca^{2+} responses was different for control and modified IS-resident Orai1-expressing cells: modified (mGFP-Orai1-Δ72 and mGFP-Orai1-Full-SAP97KD) and control (mGFP-Orai1-Full and mGFP-Orai1-Full-PURO) cells also showed a difference. While control cells (mGFP-Orai1-Full, mGFP-Orai1-Full-PURO) usually had a main peak right after the bead conjugation, which subsided over time, in mGFP-Orai1-Δ72 and mGFP-Orai1-Full-SAP97-KD cells several Ca^{2+} peaks was observed during first 60 min of IS and even after the 60th minute.

5 Discussion

5.1 The membrane expression of Kv1.3

Membrane targeting of Kv channels has been studied by multiple groups, as it is a key player in the regulation of various cellular processes including action potential regulation or immune response. To draw appropriate conclusion for trafficking it is critically important to demonstrate unambiguously that the current recorded is a consequence of the ion channel genes transfected into the cells and not influenced by endogenous K^+ channel subunits in the expression system. To do this we used kinetically or pharmacologically tagged Kv1.3 subunits with properties that uniquely distinguish homotetrameric channels formed by transfected subunits from endogenous K^+ channels or hetermultimers of endogenous and transfected channel subunits. The expression of A413V-ΔC current in CHO cells, which do not exhibit any voltage-gated K^+ conductance, supports the scenario as well that ΔC-truncated mutants are targeted to the plasma membrane without their combination with full-length Kv1.x subunits.

The outcomes with the H399K mutant also support the hypothesis that C-terminus removal downstream of the "HRET" region does not interfere with the targeting of Kv1.3 to the cell membrane. For H399K-ΔC and H399K-FL the inhibition by 100 mM TEA was negligible, which verifies that there was no mixing of H399K-ΔC and WT Kv1.3 subunits in the ER. In addition, we detected the FLAG epitopes of both A413V-ΔC and H399K-ΔC subunits in transfected cells with immunocytochemistry in non-permeabilized cells, which serves as an additional proof for their plasma membrane localization. Interestingly, we also found that A413V-NOHRET or H399K-NOHRET bearing the FLAG-epitope showed a membrane signal upon anti-FLAG staining, although patch-clamp experiments revealed that these deletion mutants produced no current. Thus, our Kv1.3 channel, even in its almost complete absence of the C-terminus (which includes the HRET sequence), is able to target into the plasma membrane (although an increase in intracellular retention of the channel protein was observed in truncated channels), but does not conduct K^+ current.

We could show, that WT Kv1.3 could not rescue the conductance of the A413V-NOHRET subunits in CHO cells, i.e., co-transfection of these two subunits resulted in pure homotetrameric WT currents. Fluorescence signals clearly showed the presence of both subunits in the plasma membrane, thus, interpretation of these results is that either WT Kv1.3 and A413V-NOHRET subunits do not form heterotetramers or that the presence of A413V-NOHRET subunits in a heteroteramer renders the channel non-conductive in a dominant negative manner. Since the cells transfected with the WT-Kv1.3 had much greater current

density as compared to cells expressing both constructs, and the inactivation kinetics of the whole-cell current in cells cotransfected is like that of the WT current, these support the formation of heterotetramers in the cell membrane. As tetramerization of Kv1.3 is governed by the N-terminal tetramerization domain, rather than the C-terminus, we favor the dominant-negative effect of the mutant subunits (as has been demonstrated for other mutations as well.

The HRET(E) is sequence is located in the part of the C-terminal that is proximal to the activation gate or may be part of it. Thus, mutation in this region may profoundly alter activation gating of the channels, the coupling of the voltage-sensor movement to the activation gate or both while leaving the voltage-sensor movement intact. To test this assumption, we determined the gating currents of WT-NOHRET channels. To our surprise, we found that majority of the cells expressing this channel did not exhibit gating currents that resemble the gating current of the non-conducting W384F mutant, which was used as a control. As fluorescence signals confirm the surface expression of the NOHRET construct this result suggests that voltage-sensor movement is impaired in the NOHRET Kv1.3 that lacks the full C-terminus including the HRET sequence. The origin of this unknown "reverse coupling" (i.e. movement of the voltage sensor is impaired by modification of the activation gate region) is unknown, but it does not seem to be specific for the HRET sequence. When we deleted just the HRETE sequence (WT-XHRETE construct) or substituted with alanines (WT-polyA) but left the rest of the carboxyl-terminus intact the conductance of Kv1.3 was recovered. So it seems that the lack of a peptide strand on the C-terminus at the activation gate renders the channels non-conductive and any replacement may substitute for the HRETE sequence, at least in Kv1.3.

5.2 The Ca^{2+} response depends on Orai1 IS localization in T cells

Secondly, we assessed the accumulation kinetics of the mGFP-Orai1 channel into the IS and its molecular background. We demonstrated that the accumulation of Orai1 channels peaked 15 min after the IS formation, then the fraction of cells showing Orai1-polarization decreased up to the 60^{th} minute. On the contrary, N-terminal truncated mGFP-Orai1-Δ72 constructs in "native" Jurkat cells and mGFP-Orai1-Full subunits expressed in STIM1/SAP97 knockdown T cells had a high fraction of Orai1-polarized cells upon engagement to CD3-CD28 beads even at the $60t^{h}$ minute. Truncation of Orai1 N-terminal region (mGFP-Orai1-Δ72) and the knockdown of SAP97 in mGFP-Orai1-Full-SAP97-KD cells had no significant effect on the Ca^{2+} current via CRAC channels. However, STIM1 knock-down abolished the Ca^{2+}-response in Jurkat cells. Ca^{2+}-signaling evoked by a CD3-CD28 bead in mGFP-Orai1-Δ72 and

mGFP-Orai1-Full-SAP97-KD cells were different as compared to corresponding control cells: the former showed persistent Ca^{2+} oscillations after 60th minute.

To understand the function of STIM1 in the synapse and Orai1 trafficking, we knocked down STIM1 in Jurkat T cells expressing Orai1-Full channels (mGFP-Orai1-Full-STIM1-KD). As expected, the STIM1 knockdown completely abolished Ca^{2+}-response of cells through CRAC channels. By monitoring the IS accumulation of Orai1 in Jurkat cells with low STIM1 expression, we could show that Orai1 is able to redistribute to the synapse even with extremely low STIM1 (knock down efficiency ca. 90%), as it was reported by Quintana et al., where overexpressed Orai1 could accumulate in the IS when exogenous STIM1 was not co-expressed. The kinetics of Orai1 redistribution in mGFP-Orai1-Full-STIM1-KD cells was different from the mGFP-Orai1-Full-PURO cells: the percentage of Orai1 polarized T cells had the maximum at 60th minute after IS formation indicating the sustained rearrangement of Orai1 channels into the contact zone between the Jurkat cells and CD3-CD28 antibody coated beads. These findings, i.e., the Orai1 redistribution to the IS does not require the calcium flux through Orai1 for targeting into the IS, and Orai1 can relocate to the IS in the absence of STIM1 are consistent with results of Lioudyno et al.: non-functional CRAC channels do not prevent IS formation and Orai1, STIM1 and TCR can redistribute to the contact zone of the immunological synapse even without the Ca2+ influx through CRAC channels.

Using the mGFP-Orai1-Δ72 mutant, we demonstrated that removal of N-terminus has a dramatic effect on the IS-accumulation of Orai1, which probably has an influence on T cell activation. Based on this, we assume that beta isoform is probably able to tune T cell activation via affecting NFAT modulation.

Based on the similar accumulation kinetics of Orai1 subunits in mGFP-Orai1-Full-STIM1-KD and mGFP-Orai1-Δ72 cells, we tested the hypothesis if SAP97 (or hDlg1), which redistributes to the IS then moves out 15 min after APC-engagement, could regulate the removal of Orai1 from the synaptic region. Upon knockdown of SAP97 we could observe (as for mGFP-Orai1-Δ72 and mGFP-Orai1-Full in STIM1-KD Jurkat cells) a continuous relocation of mGFP-Orai1-Full proteins to the IS up to the 60th minute: this points out that SAP97 and STIM1 could be crucial in regulation of Orai1 residence in the synapse.

We mentioned above that fraction of cells showing Orai1 accumulation in the IS is higher at 60 min than at 15min for mGFP-Orai1-Δ72 and mGFP-Orai1-Full-SAP97-KD. We also found that time course of Ca^{2+}-response in mGFP-Orai1-Δ72, mGFP-Orai1-Full-SAP97-KD cells is distinct from that of the controls. Though, in the first 15 and 30 min there was no difference in the Ca^{2+} response of cell lines, during the first 60 min or between the 60^{th} and the

90^{th} minute we could show significant increase in spike frequency between control and "treated" (N-terminal truncated and SAP97-KD) groups.

Thus, without either the SAP97 protein or the N-terminal part, the Orai1 channel remains functional and is able to enter and accumulate in the immunological synapse. In addition, these modifications, which result in a longer residence time, alter the time course of the Ca^{2+} signal and lead to higher and higher numbers of calcium oscillations. IS formation between the T cell and the APC can persist for up to several hours (as described in the Mark Davis Immunology Review): this time secretion of various cytokines and other factors occurs during cell-to-cell binding, which is also regulated by the cytosolic Ca^{2+} concentration. The presence of Orai1 in IS may induce persistent calcium oscillations in T cells and thereby induce pathological signaling pathways.

Finally, we suppose SAP97, which can facilitate interactions between proteins and play role in endocytosis of ion channels, may be a part of a molecular complex: Orai1 is guided out of the IS via association with STIM1 and SAP97. This presumption is consistent with the similar accumulation kinetics of the N-terminal truncated Orai1 in Jurkat cells and mGFP-Orai1-Full channels in STIM1- and SAP97-knockdown cells and the alike Ca^{2+}-spikes pattern of mGFP-Orai1-Δ72 and mGFP-Orai1-Full-SAP97-KD cells.

6 Summary

In the first part of the thesis, we demonstrated that Kv1.3 channel trafficking to the plasma membrane is preserved even if the whole C-terminus, including the HRET sequence is deleted. This finding highlights that trafficking motifs may not be universal and their importance must be tested for each channel/expression system combination. A similar conclusion can be drawn about the role of the HRET sequence in regulation of Kv1.3 channel conductance: even alanine substitution of the HRETE sequence restored channel function. Based on this and other recent papers the presence of the C-terminal amino acids adjacent to the activation gate in Kv1.3 are important for maintaining ion conductance, whereas distant C-terminal amino acids govern interactions with other proteins or confer cholesterol sensitivity to the Kv1.3.

In the second part of the thesis, we could show that the Orai1 subunit of CRAC channels have a unique accumulation kinetics in the IS. The knockdown of SAP97 and STIM1, and deletion of the putative binding site for SAP97 on Orai1 N-terminus demonstrated that SAP97 and STIM1 may be essential in the regulation of Orai1 IS-residence. Arrest of Orai1 in the IS modify the Ca^{2+} response of Jurkat T cells upon CD3-CD28 activation, which possibly contributes to T cell hyperactivity. We suppose that existence of two Orai1 isoforms could have role in regulation of T cells' Ca^{2+}-dependent activation, however, this should be elucidated. We believe that learning the molecular background of Orai1 rearrangement to the IS can facilitate the understanding the mechanism of autoimmune diseases.

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List of publications related to the dissertation

1. **Vörös, O.**, Panyi, G., Hajdu, P.: Immune Synapse Residency of Orai1 Alters Ca2+ Response of T Cells.
   Int. J. Mol. Sci. 22 (21), 1-19, 2021.
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2. **Vörös, O.**, Szilágyi, O., Balajthy, A., Somodi, S., Panyi, G., Hajdu, P.: The C-terminal HRET sequence of Kv1.3 regulates gating rather than targeting of Kv1.3 to the plasma membrane.
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List of other publications

3. Szemán-Nagy, G., Benkő, I., Király, G., **Vörös, O.**, Tánczos, B., Sztrik, A., Takács, T., Pócsi, I., Prokisch, J., Bánfalvi, G.: Cellular and nephrotoxicity of selenium species.
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