SUMMARY OF THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY (Ph. D)

Investigation of the cell surface topology and pharmacology of the human T lymphocyte Kv1.3 channel

by

Miklós Bagdány

Advisors: Prof. Dr. Rezső Gáspár and Dr. György Panyi

DEPARTMENT OF BIOPHYSICS AND CELL BIOLOGY

MEDICAL AND HEALTH SCIENCE CENTER

FACULTY OF MEDICINE

UNIVERSITY OF DEBRECEN

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1. INTRODUCTION

1.1 General characteristics of the Kv1.3 channel

The tissue distribution of voltage-gated Kv1.3 channels is restricted to the immune system and the central nervous system. The Kv1.3 channel is assembled from four identical, non-covalently linked subunits of about 500 amino acid residues. The P-loop ("pore" region, located between S5 and S6) and the S6 segment together form the ion conduction pathway. Each subunit is comprised of a hydrophobic core consisting of six transmembrane (TM) segments (S1–S6), a P-loop between S5 and S6 and long hydrophilic N- and C-termini that extend into the cytoplasm. Residues in the outer vestibule between S5 and S6 also constitute the binding site for peptide toxins and for the classical blocker tetraethylammonium (TEA). The pore region is a highly conserved sequence across the majority of K^+ channels since it contains the selectivity filter that confers the high selectivity for potassium ions over other ion species. Mutagenesis studies suggest that the S4 segment containing seven repeats of the motif Arg–X–X–Arg, along with residues in S2 and S3, constitute the voltage sensor of the channel.

The activation threshold of Kv1.3 channels is about −60mV, the open probability increases with increasing voltage and reaches saturation at −10 to 0mV. The Kv1.3 channel inactivation mechanism is the P/C-type "slow inactivation" that is different from the fast N-type inactivation present in several other voltage-gated K^+ channels. The P/C-type inactivation takes place at or near the selectivity filter as a cooperative action of the four subunits.

The lymphocyte membrane has extremely high electrical resistance (10–20GΩ). This allows the membrane potential to be regulated by a small number of ion channels. In summary, the biophysical properties of Kv1.3 channels and those of the lymphocyte membrane provide the basis for effective clamping of the membrane potential close to the activation threshold of the Kv1.3 channels.

1.2 Connection between lymphocyte activation and ion channels

Clonal proliferation of lymphocytes is a crucial event in the operation of the immune system. In this process a Ca^{2+}-dependent pathway plays an important role. For clonal expansion of lymphocytes during a general immune response, MHC

molecules of antigen presenting cells (APC) must be connected to the T-cell receptor–CD3 complex (TCR/CD3), and an immunological synapse (IS) is formed by this connection. This binding leads to the activation of protein thyrosine kinases and phospholipase-C (PLC). PLC cleaves the phosphatidyl-inositol diphosphate to inositol triphosphate (IP_3) and diacyl-glycerol (DG).

On the surface of the endoplasmatic reticulum IP_3 receptors bind IP_3 molecules, the result of which is the elevation of the intracellular Ca^{2+} level from intracellular stores and the extracellular space. The increased level of Ca^{2+} leads to the activation of the IL-2 gene. IL-2 expression is necessary for antigen independent T-cell activation. DG is also involved in the signal transduction process via the protein kinase-C (PKC) pathway. PKC eventually regulates the phosphorilation of many intracellular substrates. Kv1.3, the calcium activated IKCa1 potassium channel and the Ca^{2+}-release activated calcium channel (CRAC) have an important role in sustaining continuous calcium signal in T cells. While the liberation of Ca^{2+} in the axon terminal of neurons and myocytes is rapid, in lymphocytes it is a slow process. The increase in Ca^{2+} level is very important in antigen detection and the ability of lymphocytes to respond to the antigen.

The sustained phase of the Ca^{2+}signal is associated with Ca^{2+} selective Ca^{2+} release activated CRAC channels. Through these channels Ca^{2+} ions enter the cell following the emptying of the internal Ca^{2+} stores. Shaker-type voltage-gated Kv1.3 channels and IKCa1 channels play an important role by setting the normal resting potential of T cells to protect them against depolarization. Kv1.3 and IKCa1 channels ensure a hyperpolarized state of the cell membrane even during sustained Ca^{2+}influx.

1.3 Spatial arrangement of proteins participating in T cell activation and their connection with Kv1.3

Our knowledge about lymphocyte activation and interaction between TCR/CD3 and MHC has significantly increased by investigating the immunological synapse (IS). Between a target and a T cell an interaction takes place at a specialized intercellular contact, called immunological synapse (IS), where the encounter causes proteins to segregate into micrometer-scale domains.

Studies of the IS between T cells and antigen-presenting cells reported the formation of a supramolecular activation complex (cSMAC) with the enrichment of the lymphocyte function-associated antigen in the center, e.g. CD3, TCR ζ chain

CD28, and CD2 membrane proteins, and LFA-1 in the periphery. Similar changes to the arrangement of membrane proteins can be detected in the cytosol e.g. enrichment of talin, Lck és PKCθ. Formation of IS changes the distribution of proteins and cytoskeleton by reorganizing the lipid rafts. Spatial orientation and grouping of proteins ensure the efficiency of the TCR/CD3 complex by the result of their molecular proximity.

Different experiments suggest that Kv1.3 channels may also be participants of the immunological synapse. Most essential among these is the regulation of Kv1.3 by $p56^{lck}$ kinase during Fas receptor-mediated apoptosis of T cells. Kv1.3 channels interact with $p56^{lck}$ kinase through hDlg adaptor proteins ensuring their spatial proximity and the regulation Kv1.3 by thyrosine phosphorylation.

On the basis of the above information it was reasonable to examine the localization of Kv1.3 channels within the plain of the plasma membrane and the connection between this ion channel and the T cell receptor complex embedded in lipid rafts.

1.4 Selective blockers of Kv1.3 and IKCa1 are potential immunosuppressors

Many small non-peptide molecules (i. e. 4-aminopyridine, tetraethylammonium (TEA), correolide, progesterone, ShK-Dap22, melatonin) despite having widely differing structures and binding properties are potent blockers of the Kv1.3 ion channel. Classical channel blockers such as 4-aminopyridine, and TEA blocked lymphocyte Kv channels at micromolar to millimolar concentrations. In structure-function relation studies TEA had an important role because N- or C- type inactivation of K^+ channels can be distinguished by using TEA.

Another group of Kv1.3 channel blockers is formed by the peptide-type inhibitors. ChTX discovered in scorpion venom was the first polypeptide blocker of Kv1.3 with nanomolar affinity. Other polypeptide inhibitors such as noxiustoxin, kaliotoxin, margatoxin, agitoxin-2, hongotoxin, HsTX1, Pi1, Pi2, and Pi3 with low-nanomolar and picomolar affinities for the Kv1.3 channel were discovered in other scorpion venoms. K^+ blockers isolated from scorpion venoms contain 30-40 or 60-70 residues and are stabilized by 3 or 4 disulfide bridges. 19 subfamilies of K^+ channel blockers were defined on the basis of primary structure homology. These toxins were valuable tools in the identification of toxin-channel interaction and were used as

molecular calipers to map the pore architecture of K^+ channels. Especially ChTx was widely used for this aim including complement mutagenesis of toxin receptors and toxins. Mutagenesis studies suggested:

1, A toxin receptor is localized in the selectivity filter of S5-S6 helix close to the S5 segment.

2, The surface of the toxin interacting with the channel contains a central, positively charged amino acid residue that protrudes into the pore, and a neighboring aromatic residue placed 7Å from the α-carbon of the central lysine.

Most conclusive evidence of a connection between lymphocyte activation and K^+ ion channels was derived from pharmacological experiments. Peptide and non-peptide blockers of Kv1.3 and IKCa1 channels inhibited the sustained phase of Ca^{2+} response and proliferation of mitogen activated T cells. Inhibitors of the channels depolarized the membrane resulting in the inhibition of Ca^{2+} signaling and lymphocyte proliferation.

Recently, Chandy and his colleagues found that depending on the nature and physiological function of the T cells, i.e. naïve, central memory (TCM) and effector memory (TEM) T cells, their activation induced a specific change in the K^+ channel repertoire. Activation of effector memory T cells (TEM) was accompanied by an increase in the number of Kv1.3 channels to ~1500/cell without any change in the IKCa1 levels. The difference in the K^+ channel dominance in these T cell subsets allows specific interference with their activation using selective blockers of Kv1.3 or IKCa1 channels. Proliferation of encephalogenic TEM cells, which play a significant role in the pathogenesis of multiple sclerosis, can be suppressed by selective Kv1.3 inhibitors whereas naïve and TCM T cells escape Kv1.3 block-mediated inhibition of proliferation by up-regulating IKCa1. This selective immunosuppression places the isolation of sufficiently high specificity blockers of the T cell channels to be used in vivo with minimal side effects into the focus of ongoing research in several laboratories since these molecules are considered to have significant therapeutic potential.

2. AIMS

1, Kv1.3 channel blockers are considered to have having immunosuppressant potency with minimal side effect, therefore the search for new blockers of Kv1.3 among scorpion toxins is justified. In our experiments we were looking for the answer whether anuroctoxin isolated from Anuroctonus phaiodactylus scorpion venom was a specific and efficient blocker of Kv1.3.

2, Recently, it has been reported that voltage-gated Kv channels were differentially targeted to lipid rafts, suggesting that protein-lipid interactions should be considered as a mechanism of Kv channel localization in the plasma membrane. This observation suggested examining the cell surface distribution of Kv1.3 channels in T lymphocytes. We examined if the cell surface distribution of the Kv1.3 channels is random or even and also examined whether there is spatial co-localization between the Kv1.3 ion channels and the TCR/CD3 receptor complex playing important role in T cell activation.

3, We decided to examine the distribution of Kv1.3 channels on the surface of cytotoxic T cells in contact with a target cell, because of the importance of Kv1.3 channels in maintaining a hyperpolarized state of T cells necessary for T cell activation,. We wanted to determine if Kv1.3 channels could be found in the immunological synapse, if there was any association between the channel and signalization molecules and what were the functional consequences if such a connection existed.

3. MATHERIALS AND METHODS

3.1 Cells

In our experiments we used Jurkat lymphocytes, cytotoxic murine T cells (CTLL-2), human peripheral lymphocytes, $CD8^+$ human CTLs derived from peripheral blood mononuclear cells grown with JY lymphoblasts, L929 and B82 cell lines stably expressing mKv1.1 and rKv1.2 channels.

3.2 Scorpion toxins

Scorpion venom was obtained from animals by electric stimulation. The soluble venom was purified by high performance liquid chromatography (HPLC). Apha-2 frakcion which contained the active component was futher purified by (HPLC). Sequence of anuroctoxin was determined in a Beckamm LF 3000 analyzer, amino acid sequence by ClustalX, BLAST and FASTA searching programs. Isolation of toxins and sequence analysis were performed in our collaborants` lab (Dr. Possani, Cuernavaca, Mexikó).

3.2 Molecular biology

The FLAG epitope (DYKDDDDK) was cloned into the pRc/CMV-Kv1.3 gene in the coding sequence of the extracellular loop connecting the S1 and S2 transmembrane segments. The insertion mutation was confirmed by restriction enzyme digestion (*Aat*II) and DNA sequence analysis. Mutant channels were transferred into cells by either lipophillic reagent (Effectene or Lipofectamine), or electroporation.

3.3 Transfection of Jurkat cells

Twenty-four hours before transfection, cells were transferred to fresh media and collected in the logarithmic phase of growth. Cells were transfected by using Effectene reagent at a ratio of 1μg of DNA per 25 μl of reagent in 5.2 ml of reaction volume containing 4×10^6 cells in 60-mm Petri dishes and cultured for 24 h before immunogold or fluorescence assays.

3.4 Transfection of CTLL-2 cells

CTLL-2 cells were transiently cotransfected with plasmids encoding the WT (Kv1.3/WT) or mutant (Kv1.3/FLAG) Kv1.3 channels, along with a Ccd4neo plasmid (encoding CD4) at a 5:1 ratio using electroporation. Kv2.1 and Shaker IR channels were also transiently cotransfected by electroporation in similar conditions.

3.5 Transfection of Cos-7 cells

Cos-7 cells were transiently co-transfected with plasmids for green fluorescence protein (GFP) and for hKv1.2 at a molar ratio of 1:5 Currents were recorded 3 days after transfection. GFP positive transfectants were identified in a Nikon TE2000U fluorescence microscope.

3.6 CTL transfection and CTL-Target cell engagement

CTLs were transfected with Kv1.3/FLAG gene by using Lipofectamine 2000 reagent according to the manufacturer's instructions. Twenty-four hours after transfection, CTLs were washed in RPMI medium 1640 solution (without phenol red) and 2.5×10^5 CTLs were mixed with equal amount of JY target cells in 1 ml of complete RPMI medium 1640 (without phenol red). Cells were centrifuged (for 1 min at 200 g), and were kept at 37°C for 5 min. The conjugated cells were stored on ice and were fixed in 1% formaldehyde in PBS for 1 h.

3.7 Electrophyisiology

Whole-cell measurements were carried out by using an Axopatch 200A amplifier. T cells were selected by selective antibody adhesion. For data acquisition and analysis, the pCLAMP8 software package was used. The bath solution (in mM) was: 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.5 glucose, 10 Hepes, pH 7.35. The internal solution (mM) consisted of 140 KF, 2 $MgCl_2$, 1 $CaCl_2$, 10 Hepes, 11 EGTA except for calcium activated potassium current measurements when 150 K-aspartate, 5 HEPES, 10 EGTA, 8.7 $CaCl_2$, 2 $MgCl_2$, (pH 7.2) solution was used with 1 µM free Ca^{2+} concentration.

Blockers of Kv1.3 were added to the cells by using a gravitation perfusion system. At least three independent measurements were always performed, K_d values were calculated as mean ± SEM.

3.8 Flow cytometry measurements

Kv1.3 channels in Jurkat cell membrane were labeled at their FLAG epitopes with monoclonal anti-FLAG-M2 antibodies followed by fluorescein-conjugated rabbit anti-mouse IgG (RAMIG). Control samples were stained only by F-RAMIG. Fluorescence was detected in a Becton Dickinson FACSCalibur flow cytometer, 488 nm line was used for excitation.

3.9 Immunogold labeling

Transfected cells were labeled for transmission electronmicroscopy (TEM) measurements using anti-FLAG-M2 primer and policlonal secondary antibody conjugated with 10 or 30 nm diameter gold beads.

3.10 CLSM

In Jurkat cells CD3 molecules were labeled with Alexa Fluor 633-tagged anti-CD3 antibody and Kv1.3 channels with anti-FLAG-M2 followed by Alexa Fluor 546-tagged RAMIG. In the plasma membrane of CTLs the labeling of Kv1.3/FLAG ion channels was similar to Jurkat cells, $CD8^+$ was labeled by Alexa Fluor 647-MEM31, MHC I by fluorescein-conjugated W6/32.

Spatial localization of molecules in the plasma membrane of Jurkat and CTLs was studied by means of confocal laser scanning microscopy (CLSM). Single CLSM optical slices or projection images created from 2–3 optical slices recorded from the bottom or the top of doubly labeled cells were evaluated to determine protein colocalization. This procedure was performed by calculating the crosscorrelation between the pixel intensities of image pairs.

$$C=\frac{\sum_{i,j}(x_{ij}-<x>)(y_{ij}-<y>)}{\sqrt{\sum_{i,j}(x_{ij}-<x>)^2\sum_{i,j}(y_{ij}-<y>)^2}} \quad [1]$$

where C is correlation coefficient, x_{ij} and y_{ij} is the discrete fluorescence level of i and j pixels of x and y pictures.

4. RESULTS AND DISCUSSION

4.1 Anuroctoxin is a selective blocker of Kv1.3 channel

Our goal was to find a new specific and efficient blocker of Kv1.3 isolated from Anuroctonus phaiodactylus scorpion venom. For this we separated anuroctoxin by high performance liquid chromatography (HPLC) in several steps. Efficient Kv1.3 blocking fractions of the venom were further purified. Pure peptide subfraction with 24.72 retention time was found to be an efficient blocker of Kv1.3 and was named anuroctoxin (K_d = 0.73 nM).

The effect of anuroctoxin on Kv1.3 currents was measured in whole-cell voltage-clamped human peripheral blood T lymphocytes applied under the experimental conditions detailed in Materials and Methods. In the presence of 0.5 nM anuroctoxin approximately 50% of the channels were blocked. The block was completely reversible by perfusing the cell with toxin-free external solution. Kinetics of block and wash-out was relatively fast. 0.6 nM anuroctoxin did not change the current-voltage relationship of the Kv1.3 channels, peak currents were uniformly reduced in the presence of anuroctoxin (0.6 nM). The threshold for activation of the current was -42.5 ± 4.8 mV (n = 4) under control conditions, whereas the activation threshold was -42.5 mV ± 2.5 mV (n = 4) in the presence of 0.6 nM anuroctoxin.

The dose-response curve of Kv1.3 inhibition by anuroctoxin in the 0.05-10 nM concentration range resulted in a dissociation constant (K_d) of 0.73 nM and a Hill coefficient of 0.99. The value of the Hill coefficient indicates that a single peptide interacts with the potassium channel pore, as expected from the stoichiometry of binding of several other scorpion toxins known to block K^+ channels. On the basis of

activation, gating, voltage-independence, and the 1:1 channel-toxin binding stoichiometry anuroctoxin is a classical pore blocker of the Kv1.3 channel.

Our Mexican partners determined structure of anuroctoxin, and in the context of what is known for the other KTx's, it is clear that its structure is quite different from these other toxins. The sequence homology with all other toxins was lower than 50 percent. However, a phylogenetic cluster analysis indicated that anuroctoxin belongs to the α-KTx subfamily 6, with a systematic number α-KTx 6.12. The sequence alignment showed that K23 in anuroctoxin corresponds to K27 in ChTx, and an aromatic residue in anuroctoxin (F32) corresponds to Y36 in ChTx. Thus, the essential diad of critically positioned amino acids required for K^+ channel recognition is also found in the sequence of anuroctoxin.

Anuroctoxin was tested for specificity against some other K^+ channels. Regarding T cell physiology, the most important factor in the pharmacological profile of anuroctoxin was its selectivity for Kv1.3 over IKCa1, the Ca^{2+}-activated K^+ channel of human T lymphocytes. The applied voltage-ramp evokes pure, non-voltage-gated IKCa1 currents below the activation threshold of the Kv1.3 channels, and change in the slope of the current could be used to characterize the current block. The ratio of the slopes of the 10 nM anuroctoxin treated and control curves was 1.06 ± 0.02%. Lack of block at 10 nM anuroctoxin concentration allowed a minimal estimate of ~1000 times selectivity ratio for Kv1.3 over IKCa1.

Out of the panel of channels examined, besides Kv1.3 only Kv1.2 was blocked significantly by 10 nM anuroctoxin ($K_d \approx$ 5 nM). There was not significant blocking effect on Drosophila Shaker, mKv1.1, hKv2.1, and IKCa1 channels (remaining fractions of currents: 96.7 ± 2.68, 91.6 ± 2.11, 84 ± 1.98, 106 ± 1.7 % respectively). Several high affinity peptide blockers of Kv1.3 are selective for Kv1.3 over IKCa1, similarly to anuroctoxin (i. e. MgTX, NtX, kaliotoxin, ShK). However, ion channels important in neuronal and muscle excitability (Kv1.1, Kv1.2) are also inhibited by these toxins with nanomolar and picomolar affinities.

Nowadays many labs are searching for high specificity blockers of T cell channels having immunosuppressant effect to be used in vivo with minimal side effects. A Kv1.3-based therapy that suppresses the activation of TEM cells without significant impairment of the proliferation of naïve and TCM cells may establish a therapeutic potential for MS and other T cell-mediated immune diseases, such as

sclerosis multiplex, type I diabetes mellitus, and chronic graft rejection and sustained probably by chronically activated TEM cells.

4.2 Spatial organization of Kvl.3/FLAG channel in Jurkat cells

Cell surface distribution of voltage-dependent Kv1.3 channels of T lymphocytes was studied by using transmission electronmicroscopy (TEM) and CLSM. In order to be able to label Kv1.3 channel specifically, a FLAG epitope (DYKDDDDK) was cloned into the pRc/CMV-Kv1.3 by using PCR mutagenesis system. Mutant channels were transferred into cells by transient transfection. Transfection of Jurkat cells increased the density of the channels in the membrane to a degree where specific labeling could be performed. We proved with flow cytometry that Kv1.3/FLAG channels could be labeled specifically, the intensity of signal was enough for TEM and CLSM measurements.

CTLL-2 cells lacking endogenous voltage-gated ion channels were transfected with either Kv1.3/WT or Kv1.3/FLAG channels. Currents were evoked by depolarization to +50 mV from a holding potential of -120 mV. We found that Kv1.3 channels bearing the FLAG epitope (Kv1.3/FLAG) were functional and the biophysical parameters of the mutant current were similar to those of wild-type Kv1.3.

Based on the numerical analysis of the distribution of immuno-gold labeled FLAG epitope-tagged Kv1.3 channels we proved that Kv1.3 channels formed clusters in the membrane of Jurkat T-cells. The number of channels per unit membrane area deviated significantly from the expected values predicted by a stochastic Poisson distribution. This observation means, that the distribution of channels is not random. The nonrandom distribution of Kv1.3 channel can be explained either by protein-protein interactions between the channels and the PDZ domain containing scaffolding proteins or by protein-lipid interactions targeting the channels to specialized microdomains, termed lipid rafts, within the plane of the plasma membrane.

4.3 Colocalization of CD3 and Kv1.3/FLAG potassium channels

Because of the importance of the TCR/CD3 receptor complex in T cell activation we also examined the proximity between TCR/CD3 receptor complex and voltage-gated Kv1.3 channel.

In Jurkat cells membrane overlap of the different colors of fluorescently labeled Kv1.3 channels and CD3 molecules proved colocalization of these molecular entities. The crosscorrelation coefficient, a quantitative measure of colocalization, was calculated from the images and a fairly high correlation coefficient ($C = 0.64 \pm 0.10$ (SD), $n = 6$) was obtained, which proved the colocalization of the two proteins on a scale of a few hundred nanometers (Eq. 1). The molecular proximity indicated increases the possibility of functional connection between Kv1.3 and CD3.

4.4 Activated CTLs express a large number of voltage-gated Kv1.3 K^+ channels

Using electrophysiological experiments we proved that Kv1.3 is the native voltage-gated K^+ channel in activated CTLs. The current–voltage relationship showed that the current is activated at membrane potentials more positive than -60 mV. The midpoint and slope of the voltage dependence of steady-state activation of the whole-cell K^+ conductance was -33.8 ± 4.8 mV and 10.7 ± 0.7 mV respectively ($n = 5$). Inactivation time constant of currents was 180 ± 27 ms at +50 mV test potential ($n = 9$). Extracellular application of 10 mM tetraethylammonium increased this time constant by 56 ± 4 % ($n = 4$). These biophysical parameters are in good agreement with those reported earlier for Kv1.3 channels.

Pharmacological characterization of the current also supported this assumption. CTL potassium channels were blocked by the following toxins: MgTx (dissociation constant, $K_d = 50 \pm 12$ pM, $n = 3$), charybdotoxin (3.4 ± 0.6 nM, $n = 3$), and tetraethylammonium (14 ± 1 mM, $n = 4$), with affinities similar to those obtained for Kv1.3 expressed in lymphocytes.

4.5 Spatial rearrangement of Kv1.3/FLAG in CTLs engaged in IS

The spatial distribution of Kv1.3/FLAG in CTLs, and CTLs conjugated with target (JY) cells were studied by CLSM. Kv1.3 channels showed a patchy distribution similar to domains enriched in MHC-1 and $CD8^+$ in the plasma membrane of CTLs not in contact with target cells. In CTLs conjugated with target cells, a rearrangement of Kv1.3/FLAG was observed: in the majority of cases, ion channels were recruited into the CTL–target cell interface. In some cases Kv1.3/FLAG surrounded the IS formed between the CTL and the target cell like a belt. MHC I molecules on target

cells as well as CD8 molecules on CTLs, were also enriched in or around the contact area. The two possible arrangements of Kv1.3 in IS might show that re-arrangement of the channel is dynamic: it can be segregated in different domains of IS in the course of maturity IS.

What can be the role of the recruitment of Kv1.3 into the IS? The most important physiological function of Kv1.3 is the regulation of the membrane potential of T cells and cytotoxicity is also regulated by membrane potential. It is not likely that recruitment of Kv1.3 significantly changes local membrane potential, however kinases might phosphorylate channels concentrated in the IS more efficiently. Based on our observations it is highly possible that the activation process of the TCR/CD3 complex associated with the formation of the IS might modify the activity of a nearby ion channels thereby modulating the function of the IS and as a consequence the killing of the target cells.

5. SUMMARY

Knowledge gathered about the voltage-gated K^+ channel Kv1.3 in recent years indicates the essential role of this channel in T cell activation and the killing process of cytotoxic cells. It is therefore of utmost importance to understand the details of the physiological role of Kv1.3 in T cell signaling and the mutual interplay between the channel and other signaling molecules. The prospect of using Kv1.3 as a target for immunosuppressive drugs in therapy further emphasizes the importance of studies focusing on this channel.

As channel blockers of high affinity and selectivity are valuable tools for studying ion channel function and, in addition, they may serve as starting points in the development of new immunosuppressive agents, the search for such compounds is always justified. We identified and characterized a new peptide blocker of the Kv1.3 channel and proved that anuroctoxin selectively blocks Kv1.3 with high affinity (K_d = 0.73 nM). Regarding T cell physiology, the most important factor in the pharmacological profile of anuroctoxin is its selectivity for Kv1.3 over IKCa1, the Ca^{2+}-activated K^+ channel of human T lymphocytes. Mechanism of Kv1.3 block by anuroctoxin is similar to the block of other scorpion toxins, anuroctoxin is classical pore blocker. Because of its high selectivity for Kv1.3 (anuroctoxin did not block either Kv1.x or Kv2.1 channels), anuroctoxin might be used in clinical practice as an

effective immunosuppressive agent to block lymphocyte activation and proliferation in the future.

It is generally accepted that functional potassium channels and control of the membrane potential are essential to mitogenic induction of lymphocytes via the regulation of cell volume and cell-mediated cytotoxicity. In our experiments we examined the spatial distribution and association of Kv1.3 and molecules essential in T cell signal transduction to draw conclusions about the role of Kv1.3 in T cell activation based on these molecular interactions.

We demonstrated in Jurkat cells and CTLs that the Kv1.3 channel is raft associated and distribution of the channel is nonrandom. We demonstrated a significant domain-level colocalization of Kv1.3/FLAG and CD3 in the Jurkat cell membrane. We showed the enrichment of Kv1.3/FLAG in the immunological synapse formed by CTL-JY cells. We proved that the Kv1.3 channels enter the IS during its development and they undergo a dynamical rearrangement.

In conclusion, the results of our studies gave great contribution to the understanding of Kv1.3 function and regulation by the identification of a new high affinity and specificity blocker and the characterization of the distribution of these channels in the plasma membrane of lymphocytes.

LIST OF PUBLICATIONS AND POSTERS THE DISSERTATION IS BASED ON

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IF: 10,272
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IF: 5,650

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