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

Investigation of the inactivation and pharmacology of the human T lymphocyte Kv1.3 channel

by

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DEBRECEN, 2007

1 INTRODUCTION

1.1 Lymphocyte activation and ion channels

Clonal expansion of lymphocytes, in vivo, is required to generate an efficient generalized immune response to a specific antigen. Binding of peptide-loaded MHC of antigen presenting cell to the T-cell receptor complex (TCR/CD3) results in the recruitment and activation of protein tyrosine kinases, such as members of Src, Zap-70/Syk, Tec and Csk families of non-receptor tyrosine kinases, and the concomitant activation of phospholipase C-γ (PLCγ). PLCγ cleaves phosphatidylinositol 4,5-bisphosphate to yield diacylglycerol and 1,4,5-inositol trisphosphate (IP_3), and in turn, initiates two signaling pathways in lymphocyte activation. Diacylglycerol activates the protein kinase C (PKC) pathway, particularly through protein kinase Cθ, which leads to the phosphorylation of several intracellular substrates and the triggering of transcription via the assembly of the Fos/Jun transcription factor complex on AP1 elements in several genes. The second pathway, initiated by the generation of IP_3, governs the sustained elevation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) required for efficient signal transduction. The calcium signal activates the Ca^{2+}–calmodulin dependent phospahatase calcineurin. Calcineurin then dephosphorylates the transcription factor NF-AT thereby enabling it to accumulate in the nucleus and bind to the promoter element of the interleukin-2 (IL-2) gene. Activation of the IL-2 gene and IL-2 expression is a critical commitment point beyond which further T-cell activation becomes antigen independent. A sustained Ca^{2+} signal is required to keep NF-AT in the nucleus in the transcriptionally active state.

The first phase of the Ca^{2+} signal is directly coupled to the generation of IP_3 as IP_3 releases Ca^{2+} from endoplasmic reticulum Ca^{2+} stores via binding to the IP_3 receptor. The second, sustained phase of the Ca^{2+} signal is the consequence of Ca^{2+} entry into T cells from the extracellular space. The plasma membrane ion channel type being responsible for Ca^{2+} influx is the calcium-release activated Ca^{2+} channel (CRAC), which is activated by emptying of the intracellular Ca^{2+} stores. The current through CRAC channels is mainly determined by the electrochemical driving force for Ca^{2+}, i.e., the gating of the channel is voltage independent, but the magnitude of the current will be sensitive to the membrane potential of the cells. This, in combination with the inward rectification results in larger Ca^{2+} current at negative membrane potentials. The depolarizing inward Ca^{2+} current through CRAC channels must be counterbalanced by a cation efflux through K^+ channels in order to maintain the

electrical driving force for Ca^{2+} entry and sustain Ca^{2+} signaling required for efficient signal transduction. The membrane potential of T cells is primarily determined by the activity of two types of K^+ channels: the voltage-gated Kv1.3 and the Ca^{2+}-activated IKCa1 channel. This relationship between CRAC current and K^+ channel activity makes the proliferation of lymphocytes sensitive to pharmaceutical interference with K^+ channels. The contribution of Kv1.3 and IKCa1 channels to the membrane potential control of human T cells is not equal; it is related to the T cell subtype and the activation state of the cells.

1.2 Properties of the Kv1.3 channel

The first electrophysiological evidence about the existence of voltage-gated K^+ channels in human T cells was provided more than two decades ago. Cloning of the channel gene in 1992 allowed its classification based on homology into the *Shaker* family of voltage-gated channels (Kv1) and it has been referred to as the Kv1.3 channel.

A functional channel is composed of four identical, pore-forming alpha subunits, each subunit is composed of six transmembrane α-helices. The extracellular loop between the 5^{th} and 6^{th} transmembrane segments (S5 and S6) along with segments of the S6 helix from each subunit form the pore through which potassium ions cross the membrane. The main structural elements of the ion conduction pore are the selectivity filter, located outermost in the permeation pathway followed by a large water-filled cavity and the activation gate formed by the "bundle crossing" of S6 helices. The selectivity filter contains the signature sequence of K^+ channels. Amino acid side chains in the outer vestibule, especially in the turret region, make important contacts with peptide-toxin inhibitors of the channels thereby contributing to high affinity binding.

The activation threshold of Kv1.3 channels is between −50 mV and −60 mV, and the open probability of the channel increases steeply with depolarization. The midpoint of the voltage-dependence of steady-state activation is between −40mV and −30mV and the slope factor characterizing the steepness of the conductance-voltage relationship is around 10 mV. The voltage-dependence of steady-state inactivation of Kv1.3 has a midpoint between −60mV and −70mV and the slope factor of ~10 mV. The biophysical properties of Kv1.3 define a membrane potential "window" at which the channels can be open at steady-state. This window overlaps with the resting membrane potential of T cells, which is between −50 mV

and −70 mV, thereby underlining the importance of Kv1.3 in the membrane potential control of T cells.

Upon depolarization of the membrane Kv1.3 channels open rapidly. The activation time constant of the current becomes shorter with depolarization reaching values of 1-1.5 ms at positive voltages. The short rising phase of the whole-cell current is followed by a slow decay during prolonged depolarization as a consequence of inactivation. Members of the Shaker K^+ channel family can inactivate via two distinct inactivation mechanisms, the N-type and the so-called "slow inactivation" referring to the fact it is generally slower than the fast N-type inactivation. The Kv1.3 potassium channel lacks the N-type inactivation mechanism, so it inactivates exclusively via the slow mechanism.

Slow inactivation is believed to involve two, usually sequential, conformational responses. The first, P-type inactivation, is the closing of the gate at the extracellular end of the pore. The closure of the P-gate includes the postulated collapse of the selectivity filter (with a concomitant change in selectivity). P-type inactivation is followed by C-type inactivation, in which a further conformational change in this region stabilizes both the non-conducting (i.e., P-inactivated) state and the conformation of the voltage sensors.

The rate of slow inactivation is sensitive to the ionic composition of the extracellular solution and the type of amino acids in the pore region. It can also be modulated by channel blockers, for example tetraethylammonium (TEA), and by extracellular pH (pH_o). Raising the extracellular K^+ concentration generally slows the rate of slow inactivation, which is attributed to a "foot-in-the-door" mechanism. In high extracellular K^+ concentration the occupancy of a K^+ binding site within the pore is increased, and as a result, the structural changes leading to the inactivated state are delayed. This binding site is believed to be the outermost binding site in the selectivity filter that has been referred to as the "external lock-in site".

Mutations in the S5-S6 linker, particularly in the pore region, can alter the rate of slow inactivation. The amino acid at the position corresponding to 449 in the *Shaker* channel, which is located at the external mouth of the pore, is an especially strong determinant of the inactivation rate in various K^+ channels. Mutations at position 449 in *Shaker* produced drastic changes in the kinetics of slow inactivation.

Since the effects of changes in extracellular pH on the function of voltage-gated K^+ channels have an important physiological relevance, several studies have investigated these effects in various K^+ channels. Most studies found that lowering external pH increased the rate of slow inactivation and that pH sensitivity was enhanced by the presence of a histidine in

the S5-S6 linker region. As opposed to the other voltage-gated K^+ channels, slow inactivation of Kv1.3 is slowed in low external pH (pH_o). Furthermore, the Kv1.3 channel contains a histidine residue in the critical position of the pore region (H399, equivalent of *Shaker* 449), which makes this channel sensitive to the external pH.

1.3 Production of high affinity and high specificity blockers of Kv1.3 and IKCa1 channels: potential immunosuppressors

Based on their chemical structure molecules inhibiting Kv1.3 and IKCa1 channels are classified as inorganic ions, small-molecule inhibitors and peptide blockers. Similarly to other ion channels, peptide blockers of Kv1.3 were isolated from animals, mostly from the venom of scorpions. Scorpion toxins recognizing K^+ channels are composed of 23 to 64 amino acids. The generally accepted model is that 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 ~7 Å from the α carbon of the central lysine. This essential diad of critically spaced amino acid residues seems to be required for the recognition of K^+ channels, however, this theory has been challenged recently by the isolation of a novel scorpion toxin (Tc32) that has neither the central lysine nor the aromatic residue but blocks Kv1.3 with high affinity.

The inhibition of T-cell activation by different Kv1.3 and IKCa1 blockers raised the possibility of using these compounds as immunosuppressive agents. Koo and colleagues presented the first *in vivo* study about the immunosuppressive effects of a K^+ channel blocker in 1997.

Recently, Chandy and his colleagues found that depending on the nature and physiological function of the T cells, i.e. naïve, central memory (T_{CM}) and effector memory (T_{EM}) T cells, their activation induced a specific change in the K^+ channel repertoire. Activation of effector memory T cells (T_{EM}) resulted in the increase of expression of Kv1.3 channels to ~1500/cell without any change in the IKCa1 levels ($Kv1.3^{high}IKCa1^{low}$ channel phenotype). In contrast, naïve and T_{CM} cells respond to antigenic stimulation by an increase in the IKCa1 expression (from <30 to ~500/cell) accompanied by a modest increase in the Kv1.3 expression, therefore the channel phenotype of these cells is $Kv1.3^{low}IKCa1^{high}$. They found that in different autoimmune diseases (multiple sclerosis, type I diabestes mellitus, rheumatoid arthritis) the autoreactive T-cells are effector memory T cells (T_{EM}) with elevated Kv1.3 channel expression. The great therapeutic potential of Kv1.3-specific blockers comes

from the selective inhibition of the proliferation of T_{EM} cells while naïve and T_{CM} cells escape from Kv1.3 block-mediated inhibition of proliferation by transcriptional upregulation of IKCa1. This differential effect was shown for several Kv1.3 selective blockers. These *in vitro* results are supported by extensive *in vivo* experiments in rats. Beeton and co-workers showed that the symptoms of experimental autoimmune encephalomyelitis (EAE), a rat model for multiple sclerosis (MS) were significantly ameliorated using selective Kv1.3 inhibitors. On the basis of these results the Kv1.3 based therapy may establish a threapeutic potential for MS and other T-cell-mediated autoimmune diseases, such as rheumatoid arthritis and type I diabetes mellitus.

2 AIMS

1. Since channel inactivation modifies the total ion flux through a channel and thus has an effect on membrane potential and Kv1.3 inactivation is known to be influenced by external pH, it is important to learn about the mechanism by which inactivation gating of this channel is affected by variations of external pH. As opposed to several other voltage-gated K^+ channels, slow inactivation of Kv1.3 is slowed when external pH (pH_o) is lowered in physiological conditions, however in high extracellular [K^+] this anomalous behavior is reversed.

Our aim was to characterize the mechanism by which external protons affect slow inactivation of the Kv1.3 channel and set up a model to explain the opposite effects of extracellular acidification at different $[K^+]_e$. Furthermore, we set out to experimentally verify our model.

The principle of the model is following: The potassium ion can enter the critical binding site controlling inactivation in the mouth of the channel either from the extracellular or from the intracellular side. The protonation of His399 creates a potential barrier at the extracellular mouth of the channel, which hinders the entry of K^+ ions from the extracellular solution to the binding site and the exit of K^+ from the binding site to the extracellular space, thereby increasing the effectiveness of the filling of the binding site from the intracellular side and decreasing it from the extracellular side. To test this hypothesis we examined the combined effect of variations in extracellular pH and [K^+], introduced non-titratable neutral or positive amino acid mutations at position 399, used a high ionic strength solution to reduce the range of electrostatic interactions, measured the wash-in and wash-out kinetics of Ba^{2+} ions for wild type and mutant channels at different pH values and examined the effect of low intracellular [K^+].

2. *The immunosuppressive effect of Kv1.3 channel blockers motivated the efforts for achieving our second aim of finding or generating selective Kv1.3 inhibitors with high affinity.* In our experiments we characterized the pharmacological properties of five new peptides extracted from the venom of the Mexican scorpion *Centruroides elegans* Thorell. We examined the blocking potency of these peptides on Kv1.3, rKv2.1, *Shaker* IR and IKCa1 channels.

3 MATERIALS AND METHODS

3.1 Lymphocyte separation

Heparinized human peripheral venous blood was obtained from healthy volunteers. Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. Collected cells were washed twice with Ca^{2+} and Mg^{2+} free Hank's solution containing 25 mM HEPES buffer (pH 7.4). T lymphocytes were selected for current recording by incubation with mouse anti-human CD2 antibody resulting in selective adhesion to Petri dishes coated with goat anti-mouse IgG antibodies. Dishes were washed gently five times with 1 ml of normal extracellular bath medium (see below) before the patch-clamp experiments.

3.2 Culturing and transfection of CTLL-2 cells

Cytotoxic murine T cells (CTLL-2) were transiently co-transfected with plasmids for CD4 and for one of the hKv1.3 channels at a molar ratio of 1:5 or 1:8 (32 or 48 µg/ml total DNA) using electroporation. CTLL-2 cells were cultured in RPMI-1640 supplemented with 10% FBS, 2 mM Na pyruvate, 10 mM HEPES, 4 mM L-glutamine, 50 µM 2-mercaptoethanol, and 100 CU/ml IL-2. Before transfection, cells were cultured for 24 hours in fresh medium and collected in the logarithmic phase of growth. After harvesting, cells were suspended in Hanks'-20 mM HEPES balanced salt solution (pH 7.23) at $2x10^7$ cells/ml, and the appropriate mixture of DNA was added to the cell suspension. This suspension was transferred to electroporation cuvettes (400 µl/cuvette, 4 mm electrode gap), kept on ice for 10 minutes, and then electroporated using a BTX-electroporator with settings previously determined to give ~50% viability at 24 h post transfection (725 V/cm, 2350 µF, 13 Ω). The resultant time constants were 24-25 ms. Cells were incubated for an additional 10 min on ice, and transferred back to culture medium (~$0.5x10^6$ cells/ml) supplemented with 5 mM Na-butyrate (at 37°C, 5% CO_2). Cells were used for electrophysiology between 8-16 hours after the transfection. The selection of successfully transfected CTLL-2 cells for electrophysiological recordings was performed using a selective monoclonal adhesion strategy, as described above, but using monoclonal mouse anti-human CD4 antibody.

3.3 Culturing and transfection of HEK-293 cells

HEK 293 cells were cultured in DMEM medium supplemented with 10% fetal calf serum in a humidified CO_2 thermostate (5% CO2) at 37 °C. The cells were transiently co-transfected with plasmids for CD4 and for rat Kv2.1 (rKv2.1, kind gift from Dr. S. Korn, U.

of Connecticut) or Shaker IR channels (kind gift from Dr. G. Yellen, Harvard Medical School) using the Ca-phosphate precipitation method. The ratio of the cell surface marker gene (CD4) to the ion channel gene was 1:5 to 1:10 in order to achieve high co-transfection efficiency of the CD4 expressing cells. Ion currents were measured 2-3 days after transfection. The selection of successfully transfected cells for electrophysiological recordings was performed using a selective monoclonal adhesion strategy, as described above, but using monoclonal mouse anti-human CD4 antibody.

3.4 Molecular Biology

Plasmids (pRc/CMV backbone) encoding the wild type or the H399Y mutant Kv1.3 channel as well as a Ccd4neo plasmid containing the gene for human membrane-surface CD4 were gifts from Dr. Carol Deutsch (University of Pennsylvania. Philadelphia, PA). Other H399 mutants were generated by the use of site-directed mutagenesis *via* the QuikChange kit using the wild type Kv1.3 plasmid as a template. Mutant channels were confirmed by sequence analysis and subcloned into the original backbone in place of the wild-type channel.

3.5 Isolation of scorpion toxins

Scorpion venom was obtained from animals by electric stimulation. The soluble venom was separated by molecular sieving into a Sephadex G-50 column followed by ion-exchange chromatography into a carboxy-methyl-cellulose column (CMC). For the second chromatography only the toxic fraction to mammals (fraction II) was applied to the column. Again, the subfractions 9 to 11 (here defined II-9, II-10 and II-11) were further separated by high performance liquid chomatography (HPLC). From the resulted subfractions five independent samples were fully characterized. Automatic amino acid sequencing determination, by Edman degradation, was performed using a ProSequencer apparatus. Isolation of toxins and sequence analysis were performed in the laboratory of our collaborant (Dr. Possani, Cuernavaca, Mexico).

3.6 Electrophysiology

Whole-cell measurements were carried out using Axopatch-200 and Axopatch-200A amplifiers connected to personal computers using Axon Instruments TL-1-125 and Digidata 1200 computer interfaces, respectively. For data acquisition and analysis the pClamp6 and the

pClamp8 software package (Axon Instruments Inc., USA) were used. Standard whole-cell patch-clamp techniques were used. Pipettes were pulled from GC150F-15 borosilicate glass capillaries in five stages and fire-polished to give electrodes of 2-3 MΩ resistance in the bath. The standard bath solution (S-ECS) was (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.5 glucose, 10 HEPES (pH 7.35, 305 mOsm). The standard pipette solution was (in mM): 140 KF, 11 K_2EGTA, 1 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES (pH 7.20, ~295 mOsm). Cells with low series resistance (2-5 MΩ) were used and series resistance compensation up to 85% was applied to minimize voltage errors and achieve good voltage clamp conditions. The uncompensated series resistance error was approximately 5-10 mV. The reference electrode was connected to the recording chamber with an agar bridge to eliminate junction potential changes during perfusion.

Bath perfusion with different test solutions was achieved using a gravity-flow perfusion setup with 8 input lines and a PE10 polyethylene tube output tip with flanged aperture to reduce the turbulence of the flow. The solutions were applied in an alternating sequence of control and test solutions, unless stated otherwise. Excess fluid was removed continuously from the bath.

3.7 Data analysis

Prior to analysis whole cell current traces were corrected for ohmic leak and digitally filtered (3 point boxcar smoothing). Non-linear least squares fits were done using the Marquardt- Levenberg algorithm. Fits were evaluated visually as well as by the residuals and the sum of squared differences between the measured and calculated data points. Statistical comparisons were made using Analysis of Variance (ANOVA) supplemented with Boferroni t-test for pairwise comparisons, Student's t-test, and when appropriate, paired t-test at $p=0.05$. For all experiments, the standard error of the mean (SE) is reported.

4 RESULTS AND DISCUSSION

4.1 The role of His399 in the pH-dependent modulation of Kv1.3 inactivation

4.1.1 Low external pH slows hKv1.3 inactivation kinetics

In order to study Kv1.3 channel gating we carried out patch-clamp experiments in whole-cell configuration and votage-clamp mode on CCTL-2 cells expressing wild-type or mutant Kv1.3 channels. In accordance with earlier results, we have found that reduction of the pH of the extracellular solution reversibly reduced the current amplitude and slowed slow inactivation in the wild-type voltage-gated potassium channel Kv1.3. Because of the inherent acceleration of the rate of inactivation in whole-cell configuration, time constants were determined for control conditions both before the treatment and after the wash-out and the mean of these values was compared with the value obtained during treatment. When the cell expressing Kv1.3 channels was perfused with S-ECS pH 6.5 and 5.5 the current amplitude reduced to 91.2 ± 1.4 % and 67.1 ± 1.8 % of that measured at pH 7.35, respectively. The inactivation time constant increased to 117.2 ± 3.9 % and 138.1 ± 1.9 % of the control value in pH 6.5 and 5.5, respectively. The inactivation time constant was 167 ± 20 ms in S-ECS pH=7.35, 184 ± 25 ms in S-ECS pH=6.5 and 198 ± 23 ms in S-ECS pH=5.5.

4.1.2 Extracellular pH affects the voltage-dependence of activation

Acidification of the extracellular solution is known to induce a shift of the G-V relationship (voltage dependence of steady-state activation) toward depolarized potentials due to a surface charge screening effect. We measured this effect of acidification on Kv1.3 channels expressed in CTLL-2 cells. A Boltzmann function was fitted to the data points and the midpoint ($V_{1/2}$) and slope (s) characterizing voltage-dependence of steady-state activation were determined at different extracellular pH values. Lowering the extracellular pH induced a significant shift in $V_{1/2}$ towards depolarized voltages from $V_{1/2} = –33.6 \pm 2.6$ mV at pH 7.35 to $V_{1/2} = –20.1 \pm 2.8$ mV at pH 6.5 and $V_{1/2} = –1.5 \pm 1.6$ mV at pH 5.5. The slope factor was the same at all tested pH values (s = 6.2 ± 0.9 mV at pH 7.35; s = 7.0 ± 0.8 mV at pH 6.5; s = 6.4 ± 0.3 mV at pH 5.5).

4.1.3 Low external pH accelerates inactivation kinetics in high external potassium

Elevation of the external K^+ concentration is known to slow the rate of inactivation in various K^+ channels. This is presumably due to the higher occupancy of the external lock-in site. Moreover, the kinetics of inactivation of Kv1.3 channels becomes biphasic so current decay can be fitted by the sum of two exponential terms. The speed of inactivation can be described by two inactivation time constants, called τ_s and τ_f. When the cell was perfused with 150 mM K-ECS pH=7.35, which contains 150 mM K^+ the value of τ_f was lower than the inactivation time constant describing the inactivation in S-ECS (60.5 ± 3.8 ms), while the τ_s was a higher value: 610 ± 46 ms. The relative weight of the amplitude of the slow inactivating current component was higher than the weight of the fast inactivating component, the ratio of $A_s/(A_s + A_f)$ was 0.830 ± 0.011.

In contrast to our primary observation in S-ECS we found that in 150 mM K-ECS the inactivation of Kv1.3 current becomes faster in low extracellular pH (pH_o=6.5 and 5.5) in a dose-dependent manner. We fitted the decaying parts of current traces using a double exponential equation and we calculated the change of the amplitudes and the time constants describing the two exponential current components. At pH=6.5 the inactivation time constants did not change significantly, while at pH=5.5 τ_f increased to 127.7 ± 10.9 % of the control and τ_s did not change. The amplitudes of the individual current components changed in a dose-dependent manner, A_s decreased, A_f increased significantly at both pHs. The relative weight of the slow inactivating component significantly decreased at low extracellular pH, the value of $A_s/(A_s + A_f)$ was 0.738 ± 0.018 at pH=6.5 and 0.651 ± 0.030 at pH=5.5. The decrease of the relative weight of the fast inactivating component accounted for the faster inactivation at low extracellular pH.

4.1.4 In 40 mM K-ECS solution the inactivation kinetics does not change in low external pH

Because of the opposite effect of acidification of the extracellular solution at 5 mM and 150 mM $[K^+]_e$ we carried out the measurement also at $[K^+]_e$ of 20 and 40 mM. At these concentrations the decay of the current traces could be reliably fitted neither with a single nor a double exponential equation, so we had to use an alternative method to express the change of the rate of inactivation. We calculated the area under the leak corrected traces and we

subtracted the area corresponding to the non-inactivating current component. The ratio of this corrected area and the amplitude of the inactivating current component gives a "time constant-like" quantity, which is equal to the value of the inactivation time constant if the inactivation of current is monophasic and is inversely proportional to the overall rate of inactivation in the case of currents with biphasic inactivation kinetics. The ratio is called area/peak ratio (APPR) and thus describes the overall rate of current decay. In 20 mM K-ECS lowering the extracellular pH decreased the rate of inactivation, the APPR value increased to 120.0 ± 4.7 % of that measured at pH=7.35. In case of 40 mM K-ECS the switch from pH=7.35 to pH=5.5 did not change the inactivation kinetics, the APPR was the 97.2 ± 4.4 % of the control.

For the sake of direct comparison of the changes in inactivation kinetics at the various extracellular K^+ concentrations we calculated the APPR values for traces recorded in 150 mM $[K^+]_e$ and in S-ECS (5 mM $[K^+]_e$) solutions at different pH values and we calculated the change of inactivation kinetics in low extracellular pH based on the APPR values. The results are very similar to the ones obtained from the exponential fit indicating the reliability of this method.

4.1.5 The sensitivity of inactivation to low pH_o is related to residue H399

The most likely candidate for conveying changes in pH_o to changes in the rate of inactivation in Kv1.3 is the histidine residue at position 399 near the extracellular end of the pore. The pKa of the histidine side chain in solution is 6.0 so it is likely to change its protonation state in the examined pH range. We used TEA to assess the extent of the protonation of H399 in Kv1.3. Kavanaugh and colleagues reported that the affinity of TEA for rKv1.3 channels was greatly reduced when the histidine residue at position 401 (corresponding to H399 in hKv1.3) was protonated in low external pH. In hKv1.3 channels the extracellular application of 10 mM TEA resulted in the reduction of the current amplitude to 47 ± 3 % of the control value at pH 7.35. In contrast, the current amplitude at pH 6.5 was 94 ± 4 % of the control value in the presence of 10 mM TEA indicating a significant degree of protonation of the H399 residues.

We obtained further evidence for the key role of the protonation of H399 residues in the pH dependence of Kv1.3 inactivation by testing several H399 mutants. We replaced the titratable histidines by residues that maintain their neutral or positive charge when the pH is changed between 7.35 and 5.5, thereby isolating the effect of the charge of this residue on the rate of inactivation without the background of any possible nonspecific pH effects.

In particular, we investigated how the rate of slow inactivation is affected by extracellular acidification in H399L, H399V, H399Y, H399K and H399R. All of these mutant channels were expressed in high numbers in transfected CTLL-2 cells and were functional.

At pH 7.35 the voltage dependence of steady-state activation (G-V function) of the neutral mutants (L, Y and V) was similar to that of the wild type channel. However, the G-V function of the charged mutants (R and K) was shallower and the midpoint was shifted to more positive potentials. This indicates a possible interaction of the residue at position 399 with the activation gating mechanism of this channel. Upon exposure to a low pH solution, a shift of the G-V functions toward positive potentials was observed for each mutant, similarly to the wild type channel. In addition, the G-V curves of H399K and H399R became steeper.

Activation rates were not affected by the mutations, however, the characteristics of inactivation changed dramatically in each mutant. All the tested mutants displayed biphasic slow inactivation; the decaying parts of the current traces were well fit by the sum of two exponential terms. When cells expressing the mutant channels were exposed to solutions of pH 5.5, the overall rate of slow inactivation was accelerated in all mutants, which is in clear contrast to the behavior of the wild type channel. These results point to the role of the protonation of H399 at low external pH in producing the anomalous pH-dependence of Kv1.3 inactivation.

It is known that the rate of inactivation is controlled in many K^+ channels by the occupancy of a potassium ion binding site in the permeation pathway near the extracellular mouth of the channel. Based on our experimental results we proposed a model, which can explain the opposite effects of extracellular acidification at different $[K^+]_e$. The essence of the model is that the protonation of His399 creates a potential barrier at the extracellular mouth of the channel, which hinders the entry of K^+ from the extracellular solution to the binding site and the exit of K^+ from the binding site to the extracellular space, thereby increasing the effectiveness of the filling of the binding site from the intracellular side and decreasing it from the extracellular side. For this reason, in low $[K^+]_e$, when the site is mainly filled from the inside, K^+ ion exit is hindered, occupancy is increased and inactivation is slowed, while in high $[K^+]_e$, when filling is mainly from the outside, K^+ entry is hindered, occupancy is lowered and inactivation is accelerated.

4.1.6 Ionic interactions are involved in the slowing of inactivation at low pH_o

As a test of our hypothesis described above we used a high ionic strength extracellular solution (HIS) to decrease the effectiveness of long-range electrostatic interactions. If the positively charged side chains of the histidines are responsible for slowing the exit of potassium ions from the binding site then the effectiveness of this interaction should be reduced in a HIS solution and faster inactivation should be observed.

When the external solution was switched from S-ECS pH 7.35 to pH 5.5 inactivation was slowed, but when the cells were bathed in HIS solutions, switching from pH 7.35 to pH 5.5 caused an acceleration of the inactivation rate. When applying HIS solution to H399K at pH 7.35, which resembles the protonated state of H399, it behaved similarly to the wild type channel at pH 5.5, i.e. the inactivation rate was accelerated. However, the inactivation rate of H399L, which resembles the unprotonated state of H399, was not significantly affected by the HIS solution. These results confirm the role of electrostatic interactions and support our hypothesis.

4.1.7 Low extracellular pH slows the wash-in and wash-out kinetics of barium ions

As a further test of our hypothesis we next examined the entry and exit rates of ions into and out of the channel pore. The permeation of K^+ is too fast so we had to look for another ion, which permeates through the channel but the speed of permeation is slow enough to detect the differences in the entry and/or exit rates in different extracellular solutions. Barium ions serve as an excellent candidate for this measurement. They are able to enter the pore of different types of K^+ channels, but they are bound more strongly inside the pore because of their divalent nature. The slow permeation of barium ions hinders the permeation of potassium ions, which causes an apparent block of K^+ current.

The Shaker K^+ channels have two sequential binding sites for Ba^{2+}, a more external site, which equilibrates quickly with the extracellular solution and has a low affinity and a deep site with higher affinity and slower association and dissociation rates. The more external site, which is likely to be identical to the one termed earlier as the “external lock-in site”, is thought to be the one whose occupancy controls the rate of inactivation. According to our hypothesis, the protonation of the histidines should function as a barrier that would impede

the exit of Ba^{2+} ions from the pore to the extracellular space and the entry of barium ions into the pore of the channel from the extracellular space.

Extracellular application of 15 mM Ba^{2+} reduced K^+ currents through wild type Kv1.3 channels to 18.47 ± 1.28 % of the control. After achieving steady-state block we measured the washout kinetics of Ba^{2+} following a switch back to a barium-free extracellular solution at pH_o values of 7.35 or 5.5. Time constants of dissociation were 66.49 ± 1.17 s and 129.42 ± 8.28 s at pH_o 7.35 and 5.5, respectively.

We repeated the Ba^{2+} washout experiments with a mutant bearing a neutral residue (L) in the critical 399 position, as well as another one with a permanently positively charged residue (K). The rate of Ba^{2+} dissociation from H399L was not affected by extracellular pH and was similar to the rate of dissociation from the wild type channel at pH 7.35. Time constants of Ba^{2+} washout were also insensitive to external pH in H399K and were similar to those obtained for the wild type channels at pH 5.5.

With the protocol used in wash-out experiments the temporal resolution of wash-in was too low to detect the relatively small differences in the wash-in kinetics because steady-state block was achieved within one or two episodes. For this reason we had to modify the protocol to slow the association rate of Ba^{2+} to the channels and resolve the fast wash-in kinetics. With the modified protocol we were able to measure the wash-in kinetics and found that it was significantly slower at pH=5.5 than at pH=7.35, the time constant of wash-in was 32.8 ± 1.4 s and 46.5 ± 2.6 s at pH=7.35 and 5.5, respectively.

The wash-in kinetics of Ba^{2+} block was measured also in case of H399K and H399L mutant channels. In these experiments Ba^{2+} at 5 mM concentration caused a significantly higher block than in wild-type channels, and the wash-in kinetics was very fast, so we had to modify the modified experimental conditions to get slower wash-in kinetics. With the modified conditions we were able to determine that the wash-in was faster for H399L than for H399K. Lowering of the extracellular pH did not modify significantly the wash-in kinetics of mutant channels. These results are consistent with our expectations and confirm our model.

4.1.8 Low intracellular potassium concentration: reduced filling of the binding site from the intracellular side

We performed an additional test of our hypothesis using low intracellular potassium concentration in order to get inward potassium currents. With these conditions the filling of

the binding site from the intracellular side decreased, which increased the relative contribution of extracellular filling. Our expectation was that because of the higher contribution of extracellular filling of the binding site the protonation of the histidines at position 399 in low pH would decrease the occupancy of the critical binding site and thus would increase the rate of inactivation similarly to that seen in high extracellular $[K^+]$.

In order to be able to make direct comparisons with the datat obtained in physiological solutions we intended to keep $[K^+]_e$ at 5 mM. However, these conditions would have required intracellular $[K^+]$ so low that it would have made the channels nonfunctional. Consequently it was necessary to raise both the intra- and extracellular K^+ concentrations to evoke the inward currents. The pipette solution contained 5 mM K^+. Because of the low $[K^+]_i$ the reversal potential for K^+ differs from the conventional –85 mV. In 20, 40 or 150 mM K-ECS the calculated reversal potential for potassium was +35.4, +53.1 and +87.3 mV, respectively. During these experiments we used +20 mV test potentials instead of +50 mV to get a higher driving force and therefore a higher current amplitude. Because the +20 mV is lower than the vales of reversal potential at the used $[K^+]_e$ we get inward inactivating currents during test pulses.

At 20 and 40 mM $[K^+]_e$ the decaying part of current traces was perfectly fitted using a single exponential function, the inactivation time constants were 160.3 ± 9.6 and 199.9 ± 11.2 ms in 20 mM and 40 mM K-ECS solutions, respectively. When the external solution was switched from 20 mM K-ECS pH=7.35 to pH=5.5 solution the inactivation kinetics did not change, while in 40 mM K-ECS the same switch resulted in the acceleration of the inactivation. When the cell was perfused with 150 mM K-ECS pH=7.35 the inactivation was biphasic and application of 150 mM K-ECS pH=5.5 accelerated the inactivation kinetics. These results are consistent with our expectations and confirm our model.

4.2 Novel α-KTx peptides from the venom of the scorpion Centruroides elegans selectively block Kv1.3 over IKCa1 K^+ channels of T cells

4.2.1 Isolation and structural characterization of C. elegans peptides

Separation of soluble venom from C. elegans on Sephadex G-50 column produced at least three well-defined fractions, the only one toxic to mice was fraction II. This was further separated into a CMC-column providing 12 different subfractions. The most basic ones, II-9 to II-11 contain peptides that were recognized by mouse antibodies generated against Noxiustoxin, a well known K^+ channel blocker toxin. Three peptides were obtained in homogeneous form, from the C4 reverse-phase column, and were numbered Ce1 to Ce3, respectively. Subfraction II-10 in the same conditions provided pure peptide Ce4, whereas II-11 gave a pure peptide called Ce5. The full amino acid sequence was obtained by automatic Edman degradation.

4.2.2 Ce1, Ce2 and Ce4 toxins block potently Kv1.3 channel

The purified peptides were all tested for their blocking potency of Kv1.3 channels expressed endogenously in human peripheral blood T lymphocytes by patch-clamp. The screening was performed at 10 nM toxin concentrations. The results showed that 85% to 95% of the Kv1.3 current was inhibited in the presence of Ce1, Ce2 and Ce4, whereas the same concentration of Ce3 and Ce5 blocked less than 20 % of the current (13 ± 4 % for Ce3 and 18 ±8% for Ce5). Based on these screening data the blocking potency of Ce1, Ce2 and Ce4 was considered significant and these peptides were further investigated, whereas Ce3 and Ce5 were categorized as low affinity blockers of Kv1.3.

The equilibrium block at half-blocking concentrations of the peptides was reached within 60 s to 90 s following the start of the application of the toxin-containing solutions; full recovery from block was achieved between 180 s and 210 s following the start of the perfusion with toxin-free solutions.

We measured the blocking potency of Ce1, Ce2 and Ce4 toxins at different test potentials. All three toxins gave rise to a slightly voltage-dependent block of Kv1.3 currents in the −25 to +50 mV membrane potential range. The reduction of the peak currents was

smaller at stronger depolarization. This weak voltage-dependence of the block probably results from the interaction between the positive charges of the toxin protruding into the outer mouth of the channel and the transmembrane electric field.

For the three potent blockers a high quality dose-response curve was obtained in the concentration range of the 0.01 nM - 10 nM. The dose-response relationships were fit using a three parameter Hill equation. The resulting dissociation constants (K_d) and the Hill coefficients were 0.71 nM and 0.98 for Ce1, 0.25 nM and 0.96 for Ce2, and 0.98 nM and 0.95 for Ce4, respectively. The values of the Hill coefficients are close to 1 indicating that a single peptide interacts with the potassium channel pore, as expected from the stoichiometry of binding for several already studied K^+ channel blocking scorpion toxins.

4.2.3 Ce1, Ce2 and Ce4 toxins selectively block Kv1.3 channel

Human lymphocytes also express a Ca^{2+}-activated K^+ channel, IKCa1. As Kv1.3 and IKCa1 channel blockers differentially inhibit the proliferation of T cell subsets, it was important to determine the selectivity of these newly described Kv1.3 inhibitors against IKCa1. The applied voltage-ramps running from −120 mV to +50 mV evoke pure, non-voltage-gated IKCa1 currents below the activation threshold of the Kv1.3 channels. The block of IKCa1 currents can be determined from the change in the slope of the current traces. In the experiments we used ChTx as positive control. Application of 10 nM ChTx significantly reduced the slope of the K^+ current trace in the corresponding membrane potential range, while Ce1, Ce2 and Ce4 toxins in 10 nM concentrations were ineffective.

The selectivity of different Ce toxins was further tested against rat Kv2.1 and Drosophila Shaker IR channels expressed in HEK cells. During these experiments we used 10 nM toxin concentrations. The remaining fractions of the currents at +50 mV test potential were close to 1 in case of the Shaker IR channel, indicating the lack of inhibition of this channel by the toxins. In similar experiments using rKv2.1 channels all three toxins caused a small reduction of the current amplitude. This indicates a low affinity of the C. elegans toxins for the Shaker IR and rKv2.1 ion channels (estimated $K_d >> 100$ nM).

These results indicate that toxins Ce1, Ce2 and Ce4 are potent new blockers of the Kv1.3 ion channel of human T lymphocytes. These new toxins all show selectivity for Kv1.3 over IKCa1. Of the three effective Kv1.3 toxins, Ce2 proved to be the most potent one with a

K_d of 0.25 nM. This fact combined with the selectivity over IKCa1 may promote Ce2 to become an important tool for the modulation of the immune system, especially in cases where a selective Kv1.3 inhibitor is demanded, e.g. in the regulation of $Kv1.3^{high}$ effector memory T cell function.

5 SUMMARY

It is essential for the activation and clonal proliferation of T cells that the membrane potential required for signal transduction be provided by the K^+ conductance of the cell membrane, in which the Kv1.3 channel has a major role. The operation of the channel depends on the biophysical parameters characterizing the gating of the channel and on molecules specifically blocking the channel. Of these factors we studied the molecular mechanism of the dependence of inactivation kinetics on the extracellular pH and K^+ concentration and the Kv1.3 blocking ability and selectivity of toxins purified from the venom of the scorpion Centruroides elegans.

The Kv1.3 channel inactivates exclusively by the slow, so-called C-type mechanism, the rate of which is influenced by, among other factors, the properties of the amino acid residue at position 399,. Based on our results the reversible protonation of H399 affects inactivation via the following molecular mechanism: Through an electrostatic interaction the protonated histidines regulate the occupancy of the K^+ binding site in the selectivity filter that determines the rate of inactivation. The occupancy of the K^+ binding site is influenced by filling from both the intra- and extracellular sides. The protonation of H399 has opposite effects on these two processes: it enhances filling from the intracellular side (slowing of inactivation) while it inhibits filling from the extracellular side (acceleration of inactivation). In order to verify the validity of our model we examined the dependence of the rate of inactivation on the extracellular pH and K^+ concentration, the effect of pH_o on inactivation in solutions of various ionic strengths, as well as the kinetics of the filling of the K^+ binding site by measuring the association and dissociation rates of Ba^{2+} ions. We modeled the properties of the wild type channel containing deprotonated or protonated H399 residues with H399L and H399K mutants, respectively. Our findings about the pH_o and K^+ dependence of inactivation have significance in the understanding of the inactivation mechanism of *Shaker* K^+ channels. This mechanism may also help the adaptation of T cells to conditions in which the pH_o and K^+ concentration differ from normal conditions (e.g. in areas of inflammation).

Toxins Ce1, Ce2 and Ce4 of the five (Ce1-5) toxins purified from the venom of the scorpion Centruroides elegans that belong in the Noxiustoxin subfamily blocked Kv1.3 with high affinity without blocking IKCa1. None of the effective toxins blocked *Shaker* channels or Kv2.1 belonging to the *Shab* family. Peptides selectively blocking Kv1.3 over IKCa1 may have great significance in the therapeutical inhibition of T cell subtypes whose proliferation is dependent on Kv1.3 channels.

6 LIST OF PUBLICATIONS, ABSTRACTS AND POSTERS THE DISSERTATION IS BASED ON

Publications:

Somodi S, Varga Z, Hajdu P, Starkus JG, Levy DI, Gaspar R, Panyi G.: pH-dependent modulation of Kv1.3 inactivation: role of His399. Am J Physiol Cell Physiol. 2004 Oct;287(4):C1067-76. Epub 2004 Jun 16.

IF: 3.942 (JCR 2005)

Olamendi-Portugal T, **Somodi S**, Fernández JA, Zamudio FZ, Becerril B, Varga Z, Panyi G, Gáspár R and Possani LD.: Novel α-KTx peptides from the venom of the scorpion *Centruroides elegans* selectively block Kv1.3 over IKCa1 K^+ channels of T cells. Toxicon 2005;46:418-429.

IF: 2.255 (JCR 2005)

Somodi, S, Hajdu, P, Gaspar, R, Panyi, G, Varga, Z.: Potassium occupancy of the modulatory site regulating Kv1.3 inactivation kinetics depends on extracellular pH and potassium concentration.
(manuscript)

Published abstracts:

Somodi S, Hajdu P, Varga Z, Gáspár R, Panyi G.: C-type inactivation of Kv1.3 channels: combined effects of extracellular pH, K+ concentration and ionic strength. Biophysical Journal 84 (2): 76A-76A Part 2 Suppl. S, Feb 2003

Somodi S, Varga Z, Levy DI, Gaspar R, Panyi G.: The role of His399 in the pH-dependent modulation of Kv1.3 inactivation. Biophysical Journal 88 (1): 278A-278A Part 2 Suppl. S, Jan 2005

Varga Z, **Somodi S**, Gaspar R, Panyi G.: Effect of Extra- and Intracellular Potassium on the pH-dependent Modulation of Kv1.3 Inactivation. Biophysical Journal 90: Pos-1172, Jan 2006

Posters and lectures:

Somodi Sándor, Hajdú Péter, Gáspár Rezső, Damjanovich Sándor, Panyi György: Az extracelluláris pH és K^+ ionok hatása Kv1.3 K^+ csatornák C-típusú inaktivációjára
Meeting of Hungarian Biophysical Society, Kecskemét, 1999. (lecture)

Sándor Somodi, Péter Hajdú, György Panyi: Interaction of extracellular pH and K^+ ions with C-type inactivation of Kv1.3 channels
11th European Students' Conference at the Charité Berlin, Germany, 2000. (poster)

Sándor Somodi, Péter Hajdú, György Panyi: Interaction of extracellular pH and K^+ ions with C-type inactivation of Kv1.3 channels
Leiden International Medical Students Congress, Leiden, Netherlands, 2001. (lecture)

Sándor Somodi, Péter Hajdú, Rezső Gáspár, György Panyi: Modulation of C-type inactivation by extracellular pH and K^+ ions in Kv1.3 channel
International Summer School of Biophysics, Sovata, Romania, 2001. (poster)

Sándor Somodi, Zoltán Varga, Péter Hajdú, György Panyi: C-type inactivation of Kv1.3 channels: combined effects of extracellular pH, K+ concentration and ionic strength.
Conference of Membrane Transport, Sümeg, 2003. (lecture)

Somodi Sándor, Varga Zoltán, Hajdú Péter, Gáspár Rezső, Panyi György: A Kv1.3 K+ csatornák inaktivációjának pH-függő változása: a His399 szerepe.
Congress of Hungarian Biophysical Society, Szeged, 2003. (poster)

Somodi Sándor, Varga Zoltán, Bagdány Miklós, Gáspár Rezső, Panyi György: A Kv1.3 csatornák pH-függő inaktivációjának molekuláris mechanizmusa.
Conference of Membrane Transport, Sümeg, 2004. (lecture)

Somodi Sándor, Varga Zoltán, Bagdány Miklós, Panyi György, Gáspár Rezső: Új potenciális farmakonok limfociták káliumcsatornáinak szabályozásában.
Conference of Immunpharmacology, Debrecen, 2005. (lecture)

OTHER PUBLICATIONS, BOOK CHAPTER AND ABSTRACT:

Publications:

Szűcs A, **Somodi S**, Batta TJ, Tóth A, Szigeti PG, Csernoch L, Panyi G, Sziklai I.: Differential expression of potassium currents in Deiters cells of the guinea pig cochlea. Pflugers Arch. 2006;452:332-341.

IF: 3.564 (JCR 2005)

Hajas G, Zsiros E, Laszlo T, Hajdu P, **Somodi S**, Rethi B, Gogolak P, Ludanyi K, Panyi G, Rajnavolgyi E.: New phenotypic, functional and electrophysiological characteristics of KG-1 cells. Immunol Lett. 2004 Mar 29;92(1-2):97-106.

IF: 2.301 (JCR 2005)

Bagdany M, Batista CV, Valdez-Cruz NA, **Somodi S**, Rodriguez de la Vega RC, Licea AF, Varga Z, Gaspar R, Possani LD, Panyi G.: Anuroctoxin, a new scorpion toxin of the $\bar{t}$-KTx 6 subfamily is highly selective for Kv1.3 over IKCa1 ion channels of human T lymphocytes. Mol Pharmacol. 2004 Dec 22;

IF: 4.612 (JCR 2005)

Book chapter:

Panyi G, **Somodi S**, Varga Z, Pieri C, Pandi-Perumal SR, Damjanovich S and Gáspár R Jr.: Pharmacological effects of melatonin on ion channels. In: The pineal organ: A comparative perspective in 2000.

Published abstract:

Varga Z, Valdez-Cruz NA, Bagdany M, **Somodi S**, Gaspar R, Possani LD, Panyi G.: Anurotoxin: a potent and selective blocker of Kv1.3 channels. Biophysical Journal 86 (1): 538A-538A Part 2 Suppl. S, Jan 2004