Short Thesis for the degree of doctor of philosophy (PhD)

Pharmacological characterization of Kv1.3 inhibitor scorpion toxins

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The Examination takes place at the library of Department of Physiology, Faculty of Medicine, University of Debrecen
at 11 AM, on the 15th of September, 2014

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at 1 PM, on the 15th of September, 2014

1 INTRODUCTION

1.1 The role of ion channels in the activation of T-lymphocytes

The first ion channel described on T-lymphocytes was Kv1.3, which plays key role in the activation process of the cells. The activation threshold of Kv1.3 is -60 mV, over that membrane potential value the channel can activate thus hyperpolarizing the membrane. This hyperpolarization acts as a negative feedback on the Kv1.3 channel. The Ca^{2+}-activated KCa3.1 channel is also expressed by the cells which becomes active when the intracellular Ca^{2+}-level rises above 200 nM. The presence of other ion channels was also shown in the plasma membrane of T-lymphocytes.. These channels are the calcium release activated Ca^{2+} channels (CRAC), membrane stretch gated Cl^- channels, different TRP channels, two-pore domain K^+ channels and voltage-gated Na^+ and Ca^{2+} channels. The role of CRAC channels in the T-cell activation is well known. To activate the current through CRAC channels the association of Orai1 and STIM1 proteins is crucial. While Orai1 proteins are located in the plasma membrane STIM1 proteins are found in the endoplasmic reticulum (ER) membrane. The regulation of STIM1 upon Ca^{2+} release is through the Ca^{2+} sensor EF hands of the protein facing the lumen of the ER. Upon store release the tetramerization of ORAI1 in the plasma membrane is induced, which leads to the activation of the CRAC channels. The result of the simultaneous work of the two types of K^+ channels and CRAC is a long lasting intracellular Ca^{2+} signal which is essential for the T cell activation. During this process the driving force for the Ca^{2+} influx through CRAC is maintained by hyperpolarization through the K^+ efflux through the potassium channels.

In the last few years many peptide toxins have been isolated from animal venoms which inhibit different K^+ channels including Kv1.3 with high affinity.

For the peptide-channel interaction it is typical that the toxins bind to the extracellular region of the channels thus inhibiting ionic flux through the pore. Since the voltage-gated ion channels exhibit high sequence similarity, natural toxins generally can block more channel types thus they have low selectivity for a given channel.

Since the voltage-gated K^+ channels play key role in the regulation of cellular processes in many tissues especially in excitable cells, non-selective inhibitors have high potential risk of developing side effects upon therapeutic application.

1.2 Kv1.3 channel as a possible target in the therapy of autoimmune diseases

Since the membrane potential and the Ca^{2+} signal strongly depends on the function of K^+ channels, inhibitors of these channels can be used as therapeutic drugs to block T cell activation.

An ideal immune suppressant blocks the function of only those T cells, which are involved in the development of the autoimmune disease while the other T cells of the body remain unaffected. Such therapy can be achieved as the result of the differences in the K^+ channel expression in the different lymphocyte subtypes. Based on the cell surface markers, cytokine secretion and physiological functions three main lymphocyte groups can be distinguished.

The first group is the Naïve T cells, which have not met a specific antigen. Their specific markers are $CCR7^+$ and $CD45RA^+$. The second group is the central memory T cells (T_{CM}), which cells become inactive during the terminal differentiation progress to become effector memory T cells (T_{EM}). Repeated encounter with the specific antigen induces them to continue the differentiation process. Their markers are $CCR7^+$ and $CD45RA^-$. The third group is the effector memory T cells (T_{EM}), which have the typical markers of $CCR7^-$ and $CD45RA^{+/-}$. These cells express typical chemokine receptors and adhesion molecules, which help them to migrate to the inflamed tissues. The role of T_{EM} cells in the autoimmune tissue damage have been shown in multiple sclerosis (MS) and rheumatoid arthritis, type 1 diabetes, etc. It has also been shown that the expression pattern of K^+ channels changes characteristically in the three lymphocyte groups following stimulation. Resting Naïve, T_{CM} and T_{EM} cells independently of $CD4^+$ or $CD8^+$ phenotype express 200-300 Kv1.3 and 30 KCa3.1 channels per cell in average in their plasma membrane. Followed by activation the number of Kv1.3 channels increases to its double while the number of KCa3.1 channels increases ten-fold reaching an average value of 500 channels per cell in Naïve and T_{CM} cells. On the contrary, in T_{EM} cells the number of KCa3.1 channels per cell does not change but the level of Kv1.3 reaches the 1500 channels per cell after activation.

Since the activation process of T_{EM} cells depends on the function of the Kv1.3 channel, the inhibition of Kv1.3 can block the activation of these cells. The proliferation of activated Naïve and T_{CM} cells also depends on Kv1.3 but due to the higher expression level of KCa3.1 in the plasma membrane the activation of these cell types can be completed even in the present of Kv1.3 inhibitors. As the consequence of these mechanisms with the application of specific Kv1.3 inhibitors he activation of T_{EM} cells can be selectively inhibited thus high

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specificity Kv1.3 inhibitors can be suitable drugs in the therapy of autoimmune diseases. This hypothesis has been confirmed several times in *in vivo* autoimmune animal model systems.

T_{EM} cells with high Kv1.3 and low KCa3.1 expression level have been described in the affected tissues of human patients with different autoimmune disorders such as Multiple Sclerosis (MS), Type 1 Diabetes, (T1DM) and Rheumatoid Arthritis (RA). This makes Kv1.3 blockers suitable tools in the treatment of autoimmune disorders.

In vitro and *in vivo* experiments clearly show the efficiency and applicability of Kv1.3 blocker peptide-toxins in autoimmune therapy. Since molecules with high selectivity and affinity are isolated rarely, many research groups aimed to improve the pharmacological properties of toxin molecules with directed mutations. To design such peptides properly the detailed knowledge of the determinants of toxin-channel interactions is crucial. To gain additional information the isolation and characterization of novel peptides is a practical way.

During the previous years our workgroup participated in the isolation and pharmacological characterization of novel scorpion toxins such as Pi1, Pi2, Pi3, anuroctoxin, Css20, TsT26, Vm24. Personally I have participated in the pharmacological study of two newly isolated peptides, OcyKTx2 and urotoxin. Furthermore we conducted a detailed study on the pharmacological profile of margatoxin, which was isolated and characterized previously. Despite the fact that margatoxin is a molecule commercially available and widely used in the pharmacology of potassium channels, a detailed study of its ion channel selectivity measured in the same expressional system with the same method is not available.

Anuroctoxin (AnTx) is a peptide toxin of 35 amino acids with a molecular weight of 4082.8, stabilized by four disulfide bridges, which was isolated by our workgroup from the venom of the scorpion *Anuroctonus phaiodactylus*. AnTx is a high affinity blocker of Kv1.3 (Kd = 0.73 nM), however, with lower affinity it also blocks Kv1.2 (Kd = 6.14 nM), consequently the wild type AnTx is not suitable for therapeutic application due to its low selectivity. The comparison of amino acid sequences and the selectivity of known K^+ channel-blocking toxins provided us with the information to design mutations in anuroctoxin.

Our results provide us novel information about the toxin-channel interactions and may lead to the success of designing new peptide toxins which are high selectivity and affinity inhibitors of Kv1.3.

2   AIMS OF THE STUDY

1.   Pharmacological characterization of novel scorpion toxins

Characterization of ion channel selectivity with patch-clamp technique of novel peptides isolated from scorpion venoms.

2.   Electrophysiological characterization of the ion channel selectivity of margatoxin

We aim to screen the ion channel selectivity of margatoxin with patch-clamp technique in outside out configuration with voltage-clamp mode on the following ion channels:

Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv1.7, Kv2.1, Shaker, KCa1.1, KCa3.1, Kv11.1 (hERG) and Nav1.5.

3.   Recombinant synthesis of anuroctoxin

We aim to synthesize anuroctoxin in a biological system in *E. coli*. The structure of the toxin is confirmed by analytical HPLC and mass spectrometry (MS) methods. The biological activity is tested on Kv1.3 and Kv1.2 channels with patch-clamp technique.

4.   Solid phase chemical synthesis of wild-type and mutant anuroctoxin variants

Based on the literature we identify toxin residues that might be important factors that determine the selectivity and affinity of the toxins. With directed mutations we improve the ion channel selectivity of the toxin. The toxin variants are synthesized by solid phase chemical synthesis by our collaborators. The biological activity is tested with patch-clamp technique.

3   MATERIALS AND METHODS

3.1   Toxins

OcyKTx2 was isolated from the venom of the Brazilian scorpion *Opisthacanthus cayaporum*. Individual animals were collected then the venom was extracted with electrical stimulation. Water soluble fraction of the collected venom was separated with HPLC technique on reverse phase C18 column.

Urotoxin was isolated from the venom of an Australian scorpion, *Urodacus yaschenkoi* with similar technique as OcyKTx2.

Recombinant margatoxin (rMgTx) was purchased from Alomone Labs (Israel, Jerusalem) cat. no.: RTM-325, Lot: MA103. Margatoxin was isolated from the venom of the scorpion *Centruroides margaritatus.*

Synthetic margatoxin (sMgTx) was purchased from Peptide Institute Inc. (Japan, Osaka) cat. no.: 4290-s, Lot: 560914.

Recombinant wild type anuroctoxin (rAnTx) was synthesized in our laboratory in *E. coli* and purified with affinity chromatography and HPLC methods. Anuroctoxin was isolated from the venom of the scorpion *Anuroctonus phaiodactylus.*

Synthetic wild type anuroctoxin (sAnTx) and its mutant variants were synthesized by Prof. Gabor Toth and colleagues at the Department of Medical Chemistry, Faculty of Medicine, University of Szeged.

3.2   Cells and ion channel expression vectors

3.2.1   Human lymphocytes

The effect of the toxins on hKv1.3 channel was measured on activated human peripheral lymphocytes. Cells were isolated from the blood of healthy volunteers with Ficoll-Hypaque

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density gradient centrifugation and activated with phytohemagglutinin A. Kv1.3 currents were recorded 2-7 days after activation.

3.2.2   tsA201 cells

The following ion channel coding genes were expressed in tsA201 cells followed by transient transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA)

hKv1.1, hKv1.2, hKv1.6 and **hKv1.7** pCMV6-GFP plasmid (OriGene Technologies, Rockville, MD).

hKv1.3 (previously cloned into p-EGFP-C1 vector from pRc-CMV2 plasmid, kind gift from C. Deutsch, University of Pennsylvania, Philadelphia, PA).

hKv1.4-IR (inactivation ball deletion mutant) in pcDNA3 vector (gift from D. Fedida, University of British Columbia, Vancouver, Canada).

hKv1.5 in pEYFP vector (kind gift from A. Felipe, University of Barcelona, Barcelona, Spain)

rKv2.1 (gift from S. Korn, University of Connecticut, Storrs, CT).

Shaker-IR (inactivation ball deletion mutant) (gift from G. Yellen, Harvard Medical School, Boston, MA).

hKv11.1 (hERG, kind gift from SH. Heinemann, Max-Plank-Gesellschaft, Jena, Germany),

KCa1.1 in pClneo vector (kind gift from T. Hoshi, University of Pennsylvania, Philadelphia, PA).

KCa3.1 in pEGFP-C1 vector (gift from H. Wulff, University of California, Davis CA) and **Nav1.5** (gift from R. Horn, Thomas Jefferson University, Philadelphia, PA).

Since endogenous expression of ion channels were described in HEK cells all the electrophysiological measurements were carried out in outside-out patch configuration.

3.2.3   L929 cells

This cell line is originated from mouse connective tissue. **mKv1.1** currents were measured on L929 cells stably expressing the channel (gift of H. Wulff, UC Davis, CA, USA)

3.2.4   CHO cells

A CHO cells were used to express and measure hKv1.1 and hKv1.2.

3.2.5   Sf9 cells

Sf9, insect cells were used by our collaborators (Prof. Lourival Domingos Possani and colleagues, Instituto de Biotecnologia, UNAM, Cuernavaca, Mexico). Shaaker-IR channel was expressed in the cells following baculoviral transfection.

3.3   Transient transfection

Cells (CHO and tsA201) were transfected using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Cells were cultured under standard conditions. Vectors coding the hKv1.1, hKv1.2, hKv1.3, hKv1.5, hKv1.6 and KCa3.1 channels tag the C-terminus of the channels with GFP or YFP (green/yellow fluorescent protein) thus transfectant cells can be identified with high accuracy in fluorescent microscope.

hKv1.4-IR, rKv2.1, Shaker-IR, hERG, KCa1.1 and Nav1.5 coding vectors were co-transfected with a plasmid coding green fluorescent protein (GFP) gene in a molar ratio of 10:1. With this method cells with green fluorescence also express the ion channels with high probability.

3.4   Electrophysiology

3.4.1   Devices and solutions used for the measurements

Measurements were carried out using patch clamp technique in voltage-clamp mode. Whole cell currents were recorded on lymphocytes, CHO and L929 cells. In case of transfected tsA201 cells outside out currents were measured. For the recordings Axon Axopatch 200A and 200B amplifiers and Axon Digidata 1200 and 1440 digitizers were used (Molecular Devices, Sunnyvale, CA). Micropipettes were pulled from GC 150 F-15 borosilicate capillaries (Harvard Apparatus Kent, UK) resulting in 3- to 5-MΩ resistance in the bath solution. For most of the measurements the bath solution consisted of 145 mM NaCl, 5 mM

KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5.5 mM glucose, 10 mM HEPES, pH 7.35. For the measurements of hKv11.1 (hERG) channels the extracellular solution contained 5 mM KCl, 10 mM HEPES, 20 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM $CdCl_2$, 140 mM choline-chloride, pH 7.35. Bath solutions were supplemented with 0.1 mg/ml BSA when MgTx was dissolved in different concentrations. The measured osmolarity of the extracellular solutions was between 302 and 308 mOsM. Generally the pipette solution contained 140 mM KF, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES and 11 mM EGTA, pH 7.22. To measure Kv11.1 channels the intracellular solution consisted of 140 mM KCl, 10 mM HEPES, 2mM $MgCl_2$ and 10 mM EGTA, pH 7.22, for the KCa3.1 recordings it contained 150 mM K-aspartate, 5mM HEPES, 10 mM EGTA, 8.7 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.22 resulting in 1 µM free Ca^{2+} in the solution to activate KCa3.1 channels fully. To measure KCa1.1 the intracellular solution contained 140 mM KCl, 10 mM EGTA, 9.69 mM $CaCl_2$, 5 mM HEPES, pH 7.22 thus giving 5 µM free Ca^{2+} concentration to let the activation of KCa1.1 channels at moderate membrane depolarization. The osmolarity of the pipette filling solutions was 295 mOsM.

3.4.2   Protocols used for the measurements of ionic currents

For the measurements different voltage protocols were used. Holding potential was -100 or -120 mV depending on the cell type and the type of the measurement.

To measure Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv1.7, Shaker and Kv2.1 currents 15, 40, 50 or 200 ms long depolarization impulses were applied to +50 mV from a holding potential of -100 mV in every 15 or 30 s. Kv1.3 currents were also measured with voltage-ramp protocols where currents were elicited every 15 s with voltage ramps to +50 mV from a holding potential of -120 mV in 150 ms.

For hKv11.1 channels, currents were evoked with a voltage step from a holding potential of -80 mV to +20 mV followed by a step to -40 mV, during the latter the peak current was measured. Pulses were delivered every 30 s. For KCa1.1 channels, a voltage step to +50 mV was preceded by a 10-ms hyperpolarization to -120 mV from a holding potential of 0 mV in every 15 s. KCa3.1 currents were elicited every 15 s with voltage ramps to +50 mV from a holding potential of -120 mV. Nav1.5 currents were measured by applying depolarization pulses to 0 mV from a holding potential of -120 mV in every 15s.

3.4.3   Evaluation of the electrophysiological measurements

To acquire and analyze the measured data pClamp9/10 software package was used. The effect of the toxins in a given concentration was determined as remaining current fraction (RCF = I/I_0, where I and I_0 are current amplitudes in the presence and absence, respectively, of toxin at a given concentration). Points on the dose-response curves represent the mean of 3-8 independent measurements where the error bars represent the S.E.M. Data points were fitted with a two-parameter Hill equation, RCF = $IC_{50}^H/(IC_{50}^H+[Tx]^H$, where IC_{50} is the half inhibiting concentration H is the Hill coefficient and [Tx] is the toxin concentration. To estimate the IC_{50} using RCF of a single toxin concentration n = 1 was used for the Hill equation. Kd was also determined from Lineweaver-Burk analysis where 1/RF was plotted as a function of toxin concentration and fitting a line to the points, where IC_{50} = 1/slope.

To determine the time constant of the association (Kd) a single exponential function was fitted to the data points: $A(t) = B \times \exp(-t/T_{ON}) + C$, where $A(t)$ indicates the amplitude of the current at time t, C is the peak current at equilibrium block and $B = A(t = 0) - C$. The time constant (T_{ON}) yielded 18.6 ± 1.2 s at 10 nM urotoxin for hKv1.2, (N = 6) **(A)**, 7.9 ± 3.7 s, (n = 3) at 30 nM urotoxin for hKv1.3 and 5.7 ± 0.8 s, (N = 4) at 1 μM urotoxin for hKv1.1. The wash-out kinetics was also fitted using a single exponential function: $A(t) = B \times (1 - \exp(-t/T_{OFF})) + C$, where $B = A(t = \infty) - C$, A(t) indicates the amplitude of the measured current at time t, C is the peak current at equilibrium block.

3.5   Peptide synthesis

3.5.1   Recombinant synthesis of anuroctoxin in *E. coli*

3.5.1.1 Cloning of anuroctoxin into pPAL7 vector.

It is widely used to produce recombinant peptides in *E. coli* in the form of fusion proteins. Fusion proteins contain a fusion partner and the protein of interest in the same reading frame and produced as one protein. Fusion partners, called 'tags' (GST (glutathione-s transferase), MBP (maltose binding protein) or poly His tag) give us the ability to purify the fusion protein with affinity chromatography methods. The tag can be separated by enzymatic digestion from the protein of interest after affinity chromatography or during the process (on column cleavage) and finally a tag-free recombinant protein can be obtained

3.5.1.2 Purification of anuroctoxin with HPLC technique

Analytical examination and semi-preparative purification of anuroctoxin was done on Vydac 218TP54 0,46 X 25 cm C18 reverse phase column (Grace, Deerfield, Il, USA). Mobile phase was water-TFA/acetonitrile gradient where solution A: 0,12% trifluor acetic acid in water, solution B: 0,10% trifluor acetic acid in acetonitrile. Concentration of solution B was increased in a linear gradient from 0 to 30 % in 45 minutes.

3.5.2 Chemical synthesis of Anuroctoxin

Wild type anuroctoxin (sAnTx WT), and its mutant derivatives (sAnTx N17A, sAnTx F32T, sAnTx n17A/F32T) were synthesized by Prof. Gabor Toth and colleagues at the Department of Medical Chemistry, Faculty of Medicine, University of Szeged.

4   RESULTS

4.1   Pharmacological characterization of novel scorpion toxins

With the help of our collaboration partners our workgroup has the opportunity to take part in the characterization of the pharmacological properties of novel toxins isolated from scorpion venoms. The extraction and purification of the toxins is carried out by our collaborators while the effect of the molecules on different ion channels is mostly characterized by our workgroup with electrophysiological experiments.

In my thesis I describe the pharmacological characterization of two novel peptides, OcyKTx2 and urotoxin.

4.1.1   OcyKTx2

OcyKTx2 toxin is isolated from the venom of the Brazilian scorpion *Opisthacanthus cayaporum* by our collaborators (Prof. Elisabeth F. Schwartz, Laboratório de Toxinologia, Universidade de Brasília, Brasília, Brasília), which is a peptide of 34 amino acids with a molecular weight of 3807 Da. The tertiary structure of the molecule is stabilized by 4 disulfide bridges. The sequence of the toxin is the following:

 IRCQGSNQCYGHCREKTGCMNGKCINRVCKCYGC

(reference no.: UniProtKB/Swiss-Prot: P86116.2)

OcyKTx2 toxin inhibited Shaker and Kv1.3 channels in a reversible way. The half inhibiting concentrations were determined by both the Lineweaver-Burk and the two-parameter Hill equation methods resulting in half inhibiting concentrations on Shaker and Kv1.3 channel 93.5 nM and 18.0 nM, respectively (Lineweaver-Burk) and 96.6 nM and 17.7 nM, respectively (Hill).

The toxin had no effect on the activation threshold of Kv1.3.

Since the amount of native peptid was limited we could not test its effect on other channel types. Measurements on Shaker channel were carried out by our collaborators (Prof. Lourival Domingos Possani and his colleagues, Instituto de Biotecnologia, UNAM, Cuernavaca, Mexico)

4.1.2 Urotoxin

Urotoxin was isolated from the venom of the Australian scorpion, *Urodacus yaschenkoi*, by our collaborators (Prof. Lourival Domingos Possani, Instituto de Biotecnologia, UNAM, Cuernavaca, Mexico). The peptide consists of 37 amino acids and stabilized by 4 disulfide bridges. Its molecular weight is 4012.75 Da. The sequence of the toxin is the following:

GDIKCSGTRQCWGPCKKQTTCTNSKCMNGKCKCYGCV

The pharmacological characterization of the toxin was done on the hKv1.1, hKv1.2 hKv1.3, hKv1.5 and KCa3.1 channels. Measurements on hKv1.1 were carried out by our collaborators (Prof. Lourival Domingos Possani, Instituto de Biotecnologia, UNAM, Cuernavaca, Mexico). The toxin blocked hKv1.1 channels expressed in CHO cells reversibly. The half inhibiting concentration was determined by the two parameter Hill equation resulted in IC_{50} = 253.5 nM. Since Kv1.1 shows high sequence homology to Kv1.2 and Kv1.3 we tested the effect of the peptide on these channels as well. 10 nM toxin inhibited the Kv1.2 channel dramatically therefore with further measurements in lower concentrations we determined the half inhibiting concentration, which was 160.5 pM.

Urotoxin also blocked Kv1.3 however with lower affinity. Half inhibiting concentration was determined with the Lineweaver-Burk method which gave a result of IC_{50} = 90.9 nM.

Since well known inhibitors of Kv1.2 such as charybdotoxin and maurotoxin block the KCa3.1 channel as well we tested urotoxin on the channel. Urotoxin blocked hKCa3.1 channel in 10 nM reversibly. To determine the half inhibiting concentration we conducted measurements in higher concentrations (30 and 100 nM). Urotoxin blocked hKCa3.1 with IC_{50} = 69.9 nM.

We measured the effect of urotoxin on hKv1.5 but 10 nM toxin had no effect on the channel.

4.2 Measuring the selectivity of margatoxin with electrophysiological methods

Recombinant margatoxin (rMgTx, Alomone Labs, Israel, Jerusalem) was tested on mKv1.1, hKv1.1, hKv1.2, hKv1.3, hKv1.4, hKv1.5, hKv1.6, hKv1.7, rKv2.1, Shaker-IR, hKCa1.1, hKCa3.1, hKv11.1 (hERG) and hNav1.5 channels.

Since margatoxin (rMgTx) in 1 nM concentration blocked the mKv1.1, hKv1.1, hKv1.2 and hKv1.3 channels, further measurements were carried out to determine the half inhibiting

concentrations on these channels. The effect on Kv1.1 channels was lower, margatoxin blocked mKv1.1 with $IC_{50} = 1.7$ nM, hKv1.1 with $IC_{50} = 4.7$ nM In case of hKv1.2 and hKv1.3 channels 1nM margatoxin completely inhibited the currents, the half inhibiting concentrations were 6.4 pM and 11.7 pM, respectively.

4.3   Effect of the recombinant anuroctoxin (rAnTx) on Kv1.2 and Kv1.3 channels

Effect of the recombinant anuroctoxin (refolded, rAnTx) was measured on Kv1.3 and Kv1.2. The half inhibiting concentrations were 0.2 nM and 1.4 nM for Kv1.3 and Kv1.2 channels, respectively.

4.4   Solid phase chemical synthesis of wild type and mutant anuroctoxin variants

4.4.1   Designing the mutations to improve the selectivity of anuroctoxin

To design the mutations we compared the linear amino acid sequences of anuroctoxin and other toxins with known ion channel selectivity. Based on the typical common amino acid residues observed we designed the following mutations: N17A, F32T and a double mutant including both mutations N17A/F32T.

Wild type anuroctoxin and its mutant derivatives were synthesized by Prof. Gabor Toth and colleagues at the Department of Medical Chemistry, Faculty of Medicine, University of Szeged.

4.4.2   Effect of the wild type and mutant anuroctoxin variants produced by chemical synthesis on Kv1.2 and Kv1.3 channels

Synthetic wild type anuroctoxin (sAnTx WT) blocked Kv1.2 channel with $IC_{50} = 5.2$ nM and Kv1.3 with $IC_{50} = 0.3$ nM. These values match well with the values of the native anuroctoxin where $IC_{50} = 6.1$ nM (Kv1.2) and $IC_{50} = 0.7$ nM (Kv1.3).

Exchange of phenylalanine in position 32. to treonine (F32T) decreased the affinity to Kv1.3 $IC_{50} = 6.2$ nM, but the toxin lost its affinity to Kv1.2 as it was not effective in 100 nM.

The exchange of asparagine in position 17. to alanine (N17A) decreased the affinity to both Kv1.3 and Kv1.2 to the same extent $IC_{50} = 1.2$ nM and 20.0 nM, respectively. The mutation did not affect the selectivity.

The double mutant toxin (N17A/F32T) blocked Kv1.3 with $IC_{50} = 0.6$ nM and did not inhibit Kv1.2 in 100 nM.

4.4.3   Effect of the wild type and mutant anuroctoxin variants produced by chemical synthesis on Kv1.1 and KCa3.1 channels

Since the applied mutations changed the affinity of anuroctoxin to Kv1.2 and Kv1.3 we tested the toxins on Kv1.1 and KCa3.1 channels as well since mutations may develop new binding capabilities to other potassium channels. Applying the toxins in 100 nM we did not observe blocking effect on hKv1.1 and hKCa3.1 channels.

5   DISCUSSION

5.1   Pharmacological characterization of novel scorpion toxins

With the isolation and characterization of OcyKTx2 we described a novel potassium channel blocker scorpion toxin which has the systemic name α-KTx6.17. Since the amount of natural toxin was limited we could not characterize its pharmacological properties completely. OcyKTx2 inhibited Shaker and Kv1.3 channels. Comparison of the linear amino acid sequence of the toxin with other toxin's with known ion channel selectivity could give the possibility to estimate possible further targets of the toxin. Since OcyKTx2 shows lower sequence similarity to other toxins with known selectivity we could not identify further possible targets of the peptide with this method.

Based on sequence homology urotoxin was classified as α-KTx 6.21according to the nomenclature of K^+ channel blocking scorpion peptides. We could measure the activity of urotoxin on many different ion channels with electrophysiological methods. The toxin was not effective on Kv1.4, Kv11.1, Kv12.2 (measurements were carried out by our collaborators) and Kv1.5 channels.

The peptide inhibited Kv1.2 with high affinity ($IC_{50} = 160$ pM), and with much lower affinities the Kv1.1, Kv1.3 and KCa3.1 channels were also blocked. It is commonly accepted that aa toxin can be considered a selective inhibitor if it blocks a given channel with at least

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100 fold higher affinity than other channels. Urotoxin meets this criteria therefore we can consider urotoxin a high affinity and selective inhibitor of Kv1.2 since the half inhibiting concentrations on other channels measured show at least 400 fold differences ($IC_{50}Kv1.3/IC_{50}Kv1.2$ = 562, $IC_{50}Kv1.1/IC_{50}Kv1.2$ = 1579 and $IC_{50}KCa3.1/IC_{50}Kv1.2$ = 435). Comparing the pharmacological properties of urotoxin to maurotoxin, a well known and widely used inhibitor of Kv1.2 (IC_{50} = 0.8 nM) we can state that urotoxin is a higher affinity inhibitor of the channel. Moreover, maurotoxin inhibits the Kv1.1 channel IC_{50} = 45 nM, Kv1.3 channel IC_{50} = 180 nM and the KCa3.1 channel IC_{50} = 14 nM thus in case of maurotoxin the criteria of selectivity to Kv1.2 does not meet the requirements ($IC_{50}Kv1.3/IC_{50}Kv1.2$ = 225, $IC_{50}Kv1.1/IC_{50}Kv1.2$ = 56 and $IC_{50}KCa3.1/IC_{50}Kv1.2$ = 18).

Consequently urotoxin can be a useful tool in the pharmacological studies of Kv1.2. Since the role of Kv1.2 was shown in the decreased signal transmission in demielination illnesses urotoxin can be useful in the studies aiming the treatment of such diseases.

5.2   Measuring the selectivity of margatoxin

Linear amino acid sequence of margatoxin shows the highest sequence homology (>75%), which inhibit both Kv1.3 and Kv1.2 with similar affinities. We examined the effect of margatoxin on several channels (mKv1.1, hKv1.1, hKv1.2, hKv1.3, hKv1.4-IR, hKv1.5, hKv1.6, hKv1.7, rKv2.1, Shaker-IR, hKv11.1, hKCa1.1, hKCa3.1 and hNav1.5) and we found that the toxin inhibits Kv1.1 in nanomolar concentration and the Kv1.2 and Kv1.3 channels in picomolar concentrations.

Our results show that the margatoxin is indeed a high affinity inhibitor of Kv1.3 however it is not selective to the channel since it blocks Kv1.2 with similarly high affinity and Kv1.1 with lower but still significant affinity. The toxin does not meet the criteria of selectivity because the relation of half inhibiting concentrations ($IC_{50}Kv1.2/IC_{50}Kv1.3$ = 0.54) does not reach the 100 fold difference.

5.3   Synthesis and pharmacological characterization of wild type and mutant anuroctoxin variants

We produced the wild type anuroctoxin with recombinant technique expressed in *E. coli* cells followed by affinity chromatography purification. The analytical examination of the peptide

showed that the oxidation of the toxin in prokaryotic cells is not proper. Refolding the sample is necessary for the desired biological activity on Kv1.3 since the effect of the peptide before refolding is far lower (IC_{50} = 65.9 nM) than that of the natural anuroctoxin (IC_{50} = 0.7 nM). Reduction and oxidation of the sample greatly increased the effect on Kv1.3 (IC_{50} = 0.2 nM) thus we determined the dose dependence of the inhibition on Kv1.2 as well with a result of IC_{50} = 1.4 nM which in case of natural anuroctoxin was IC_{50} = 6.1 nM.

Comparing the effect of recombinant and natural anuroctoxin we can conclude that the effect of the peptides are within the same order of magnitude therefore the synthesis is suitable for the production of peptide toxins.

Efficiency of the wild type anuroctoxin produced by solid phase chemical synthesis was also measured with patch-clamp technique. The biological effect of synthetic wild type anuroctoxin (sAnTx WT) is similar to that of the natural toxin therefore the efficacy of the synthesis is proper for such peptides.

Phenylalanine in position 32 is similar in structure to tyrosine as both have aromatic side chains. Most of the peptides with affinity to Kv1.2 possess tyrosine in the equivalent position however Kv1.3 selective toxins have treonine or asparagine. In the case of anuroctoxin the substitution of phenylalanine to treonine in position 32. dramatically increased the selectivity of the peptide to Kv1.3 however the somewhat decreased the affinity to the channel compared to the wild type toxin.

In position 17. anuroctoxin has a polar side chain residue, asparagine. In many cases Kv1.2 inhibitors have the polar glutamine or the positively charged arginine in the equivalent position of their sequences. On the contrary the hydrophobic alanin is typical for Kv1.3 selective peptides. Although with the mutation N17A we expected the improvement of the selectivity to Kv1.3, this parameter of the peptide did not change but the affinities to Kv1.2 and Kv1.3 both decreased slightly. This decrease in the affinity was not as significant as in the case of F32T mutant.

In the case of the simultaneous application of both mutations we expected to get a toxin which has a high selectivity to Kv1.3 due to the mutation F32T and has an affinity closer to the wild type toxin by the mutation N17A. Fulfilling our expectations the double mutant anuroctoxin (N17A/F32T) blocked the Kv1.3 selectively and with similar affinity as the wild type molecule.

Based on its selectivity and affinity to Kv1.3 the N17A/F32T double mutant toxin can be a suitable tool in *in vitro* and *in vivo* experiments that aim the treatment of autoimmune diseases. Our further goal is to determine and compare the exact NMR solution structures of the wild type and mutant toxins for the better understanding of the molecular elements that determine the selectivity of the toxin.

The comparison of the linear amino acid sequences and pharmacological parameters of the peptides which were presented in my work underlines the statement that the essential dyad plays key role in the potassium channel binding ability of the toxins. OcyKTx2, urotoxin, margatoxin and anuroctoxin all possess amino acid with aromatic side chain in the dyad together with the lysine. All of them except OcyKTx2 block both Kv1.2 and Kv1.3 channels which may suggest that OcyKTx2 is also a possible inhibitor of Kv1.2.

The substitution of the aromatic residue of the dyad to treonine in the case of anuroctoxin resulted in the loss of affinity to Kv1.2 while the peptide could inhibit Kv1.3 with nanomolar affinity. As the conclusion of our findings we can state that the role of the essential dyad is not only the establishment of the general connection between the channel and the peptide but also an important determinant of the ion channel selectivity of the peptides.

6   Summary

The Kv1.3 channel plays a key role in the activation process of effector memory T-cells (T_{EM}), which are the main causatives of various autoimmune diseases. Inhibition of the K^+ current through the channel can block the activation of T_{EM} cells. The potential of Kv1.3 inhibitors in the treatment of autoimmune diseases has been shown in several *in vitro* and *in vivo* experiments.

We investigated toxins of 30-40 amino acids stabilized by disulfide bonds isolated from scorpion venoms, which are high affinity inhibitors of different K^+ channels.

The aims of our research were to isolate and characterize new scorpion toxins, to perform a detailed screening of the selectivity of the previously isolated and characterized margatoxin, to synthesize the scorpion toxin, anuroctoxin and to improve its pharmacological properties with directed mutations. The effect of toxins on the ion channels was measured with electrophysiological measurements using the patch-clamp technique.

With the help of our collaboration partners we could isolate two novel scorpion toxins, OcyKTx2 (α-KTx 6.17), which is a non-selective inhibitor of *Shaker* and Kv1.3 channels and urotoxin (α-KTx 6.21), which is a high affinity and selective inhibitor of Kv1.2. Margatoxin is considered a high affinity and selective inhibitor of Kv1.3. Our results show that margatoxin blocks the Kv1.3 and Kv1.2 channel with similarly high affinity (pM concentration) and the Kv1.1 channel with lower affinity (nM concentration). With recombinant technique and solid phase chemical synthesis we could successfully synthesize the previously characterized anuroctoxin. This peptide naturally inhibits both Kv1.2 and Kv1.3 channels which is not advantageous for the therapeutic application in autoimmune diseases. With site directed mutations we could design and synthesize a variant of anuroctoxin, which is a high affinity and selective inhibitor of the Kv1.3 channel.

As the result of our work with scorpion toxins, which inhibit Kv1.3 we could identify amino acid residues that influence the affinity and selectivity of toxins for Kv1.3. Our results provide us valuable data to understand the interactions between toxins and ion channels, which is crucial for the design and synthesis of future peptides highly selective for Kv1.3 in the therapy of autoimmune diseases.

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List of publications related to the dissertation

1. **Bartók, Á.**, Tóth, Á., Somodi, S., G. Szántó, T., Hajdú, P., Panyi, G., Varga, Z.: Margatoxin is a non-selective inhibitor of human Kv1.3 K+ channels.
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