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

Molecular determinants of toxin selectivity of Kv1.2 and Kv1.3 K^+ channels

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1. INTRODUCTION

1.1 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.

1.2 General characteristics of the potassium channel

The voltage-gated K^+ channel (Kv) 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 physiological function of Kv1.3 channel is to regulate the membrane potential and the Ca^{2+}-signaling. This is the typical voltage dependent channel of the immune system and the central nervous system. But it occurs in the Brain, lung, islets, thymus, spleen, lymph node, fibroblasts, B and T lymphocytes, pre-B cells, tonsils, macrophages, microglia, oligodendrocytes, osteoclasts, platelets, testis. It can coassemble with other Kv1 family members in heteromultimers but not with members of other Kv families.

The physiological role of Kv1.2 channel is to maintain membrane potential and to modulate electrical excitability in neurons and muscle. It is present furthermore in the brain, spinal cord, Schwann cells, atrium, ventricle, islet, retina, smooth muscle, PC12 cells. This channel can also coassemble with other Kv1 family members in heteromultimers but not with members of other Kv families.

1.3 Selective profile of toxins

Scorpion venoms contain a complex mixture of peptides, many of which can bind to ion channels with great affinity. Over 120 of these are known to block K^+ channels, being more or less selective among different K^+ channels. Many of these toxins share a very similar structure having an α-helix and two β-strands linked by three disulfide bridges. A strategically positioned lysine and an aromatic residue nine positions downstream form the “functional dyad”, which was also identified as a common feature of high affinity K^+ channel blocking scorpion peptides. The aromatic residue, however, is not a prerequisite for effective block of K^+ channels in general; it rather seems to determine selectivity among channel subtypes, such as Kv1.2 and

Kv1.3. Moreover, scorpion toxins completely lacking this dyad, yet effectively blocking potassium channels also exist. Although the side chain of the dyad lysine that protrudes into the selectivity filter of K^+ channels is very important for high affinity block, a long lasting toxin-channel complex requires multiple contact points within the interacting surfaces. Differences in these contact points determine the selectivity of the toxin among different channels. High selectivity for one particular channel type is of utmost importance if a toxin or its derivative is to be used in therapy in the future to influence a specific function of the targeted cells. Thus, the comparison of the selectivity of K^+ channel specific toxins with known structures is needed to identify key residues making contacts with the channels. This information, combined with molecular biological techniques then can be used to produce compounds that target K^+ channels with high affinity and selectivity to achieve the desired therapeutic effect.

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.

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. 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.

2. AIMS

- **To discover and to characterize new Kv1.3 channel inhibitor scorpion toxins with high affinity.**

  We got isolated peptides from the venom of Centruroides suffusus suffuses and Tityus stigmurus scorpion to analyze.

- **To examine the selectivity of new effective (Kv1.3 blocker) toxins (Css20 and Tst26).**

  To check the blocking effect of these toxins on different voltage gated (mKv1.1, hKv1.2, hKv1.4, hKv1.5, hBK, hERG, hK2.1) and calcium activated (hIKCa1) potassium channels together with a heart muscle sodium channel (hNav1.5).

- **To determine the pharmacological features of these toxins for the inhibited channel types (Kv1.2 és Kv1.3).**

  To prepare dose-response curve, to determine the K_d and the Hill-coefficient for both toxins for both channels.

- **To examine the biophysical properties of Kv1.2 and Kv1.3 channel during the application of these toxins.**

  To examine the steady-state activation and inactivation. To determine the value of $V_{1/2}$ for both toxins for both channels regarding to activation and inactivation.

- **To analyze the possible determinants of selectivity for Kv1.2 and Kv1.3 channel.**

  To analyze amino acid sequence: comparison with other effective toxin sequence, with the results of toxin mutation experiments and with the results of docking simulations.

3. MATHERIALS AND METHODS

3.1 Scorpion toxins

Scorpion venoms were obtained from animals by electric stimulation. Css20 and Tst26 toxins came from *Centruroides suffusus suffuses* and *Tityus stigmurus* scorpion, respectively. The soluble venoms were purified by high performance liquid chromatography (HPLC). Frakcion which contained the active component was futher purified by HPLC (Css20 and Tst26 refer to the 20^{th} and 26^{th} fractions). Sequences were determined in a Beckamm LF 3000 analyzer. Isolation of toxins and sequence analysis were performed in our collaborants` lab (Dr. Possani, Cuernavaca, Mexico).

3.2 Cells

3.2.1 Lymphocyte separation

Kv1.3 currents were measured in human peripheral T lymphocytes. 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). Cells were cultured in a 5% CO_2 incubator at 37 ºC in 24 well culture plates in RPMI-1640 supplements with 10% FCS (Sigma–Aldrich, Hungary) 100 mg/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine at 0.5 x 10^6/ml density for 3–4 days. The culture medium also contained 2.5 or 5 mg/ml of phytohemagglutinin A (PHA-P, Sigma–Aldrich Kft, Hungary) to increase K^+ channel expression.

2.3.2. Heterologous expression of channels

Cos-7 cells were transiently transfected with the plasmid for hIKCa1 (subcloned into the pEGFP-C1 (Clontech) in frame with green fluorescence protein (GFP)); or co-transfected with plasmids for GFP and for hKv1.2 (pcDNA3/Hygro vector containing the full coding sequence for Kv1.2); or hKv1.4 (hKv1.4DN: the inactivation ball deletion mutant of Kv1.4); or rKv2.1; or hNav1.5. hBK (hSlo1 gene (U11058), in pCI-neo plasmid) and hKv1.5 channels were transiently co-transfected into tsA-201 cells along with plasmids encoding GFP.

Transfections were done at a GFP:channel DNA molar ratio of 1:5 using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA), and cultured under standard conditions. Currents were recorded 1 day after transfection. GFP positive transfectants were identified in a Nikon TE2000U fluorescence microscope. More than 70% of the GFP positive cells expressed the co-transfected ion channels.

Cos-7 cells were maintained in standard cell culturing conditions. Human embryonic kidney cells transformed with SV40 large T antigen (tsA201) were grown in Dulbecco's minimum essential medium–high glucose supplemented with 10% FBS, 2mM $_{L}$-glutamine, 100 U/ml penicillin-G, and 100 mg/ml streptomycin (Invitrogen) at 37 ºC in a 9% CO_2 and 95% airhumidified atmosphere. Cells were passaged twice per week after 7-min incubation in Versene containing 0.2 g/L EDTA (Invitrogen).

hERG channels were expressed in a stable manner in a HEK-293 cell line. L929 cells stably expressing mKv1.1- channels have been described earlier [15] and were gifts of H. Wulff (UC Davis, CA, USA).

3.3 Electrophyisiology

Whole-cell measurements were carried out by using an Axopatch 200A and Multiclamp 700B amplifier connected to a personal computer using Axon Digidata 1200 and 1322A data acquisition hardware, respectively (Molecular Devices Inc., Sunnyvale, CA). Series resistance compensation up to 70% was used to minimize voltage errors and achieve good voltage-clamp conditions. Cells were observed with Nikon TE2000-U or Leitz Fluovert fluorescence microscopes using bandpass filters of 455–495 nm and 515–555 nm for excitation and emission, respectively. Cells displaying strong fluorescence were selected for current recording and >70% of these cells displayed co-transfected current. Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries in five stages and fire-polished, resulting in electrodes having 3–5 MV resistance in the bath. The external solutions were supplemented with 0.1 mg/ml bovine serum albumin to avoid toxin binding to the plastic surfaces. Bath perfusion around the measured cell with different test solutions was achieved using a gravity-flow perfusion system. Excess fluid was removed continuously.

3.4 Data processing

For data acquisition and analysis, the pClamp8/10 software package (Molecular Devices Inc., Sunnyvale, CA) was used. Before analysis, whole-cell current traces were corrected for ohmic leakage and digitally filtered (three-point boxcar smoothing). Each data point on the concentration–response curve represents the mean of three to seven independent experiments, and error bars represent S.E.M. Data points were fitted with a two parameter Hill-equation: $RCF = \frac{K_d{}^n}{K_d{}^n + [Tx]^n}$, where RCF is the remaining current fraction ($RCF = \frac{I}{I_0}$, where I and I_0 are the current amplitudes in the presence and absence of the toxin of given concentration, respectively), K_d is the dissociation constant, n is the Hill coefficient and $[Tx]$ is the toxin concentration.

The voltage dependence of steady state activation and inactivation was characterized by the parameters of fitted Boltzmann-function:

$$G_{norm} = \frac{1}{1 + e^{-\frac{V - V_{1/2}}{k}}} \text{ and } \frac{I}{I_{-120}} = \frac{1}{1 + e^{-\frac{V - V_{1/2,i}}{k_i}}},$$

where G_{norm} and I/I_{-120} are calculated from the measured data, V is the test potential, $V_{1/2}$ and $V_{1/2,i}$ are the half activation and half inactivation potentials respectively, k and k_i are the slopes of the voltage dependence of activation and inactivation.

4. RESULTS AND DISCUSSION

4.1 Selectivity of Css20 and Tst26

We tested the effect of Css20 and Tst26 on different potassium channels and a voltage-gated sodium channel by measuring whole-cell ionic currents using the patch-clamp technique. Among the tested channels were five members of the voltage-gated Shaker family, Kv1.1, Kv1.2, Kv1.3, Kv1.4 and Kv1.5. In addition, IKCa1 ($K_{Ca}3.1$), an intermediate conductance Ca^{2+} activated potassium channel, BKCa ($K_{Ca}1.1$), a big conductance voltage and Ca^{2+} activated potassium channel, Nav1.5, a cardiac sodium channel were also tested and Kv2.1 (but this latest was tested by only Css20). These channels were expressed by various cell lines and appropriate voltage protocols were applied to evoke ionic currents (for details see Materials and Methods), then the toxin was applied at a concentration of 10 nM to check for any blocking effect. There were only two channels that were blocked by the toxins: Kv1.2 and Kv1.3. The application of 10 nM Tst26 reduced Kv1.2 currents by 84%, Kv1.3 currents by 47%, whereas the reduction of the currents through other channels was less than 6%. Furthermore 10 nM Css20 reduced Kv1.2 currents by 93%, Kv1.3 currents by 58%, whereas the reduction of the currents through other channels was less than 7%. It means that the estimated K_d value greater than 150 nM for the others channels.

Thus, Tst26 and Css20 are selective blockers of Kv1.2 and Kv1.3 over the other channels examined in this study.

4.2 Pharmacological properties of Css20 and Tst26 on Kv1.2 and Kv1.3 channels

In order to better characterize the effect of Css20 and Tst26 on Kv1.2 and Kv1.3 channels we conducted a more detailed study including testing at various toxin concentrations, measuring the association and dissociation kinetics and examining steady-state voltage dependent parameters.

Kv1.2 currents were measured in whole-cell voltage-clamped Cos-7 cells. After the addition of 3 nM Css20 and 1 nM Tst26 to the external solution approximately 75% and 40% of the Kv1.2-channels were blocked, respectively. The block was partially reversed in the case of Css20 and completely reversed for Tst26 by perfusing the cell with toxinfree external solution. The association and dissociation kinetics of both toxins were slow, several minutes were required to reach steady-state

block and many minutes of wash-out with toxin-free solution resulted in recovery from block.

Both toxins were less effective on Kv1.3-channels. Under the experimental conditions applied to human peripheral blood T cells the whole-cell currents recorded were conducted exclusively by the endogenously expressed Kv1.3-channels. Extracellular application of 10 nM Css20 and 10 nM Tst26 reduced the whole-cell K^+ current by approximately 60% and 50%, respectively. The blocks of Kv1.3-channels by the toxins were fully reversible and had very fast kinetics. Both the equilibration of the block and full relief from the block took place between two subsequent depolarizing pulses separated by 30 s.

The concentration–response functions of Css20 and Tst26 on both Kv1.2- and Kv1.3-channels were constructed by determining the remaining fraction of the whole-cell current in the presence of various concentrations of the toxin. The remaining current fractions (RCF) were calculated as I/I_0 where I_0 and I are the peak K^+ currents measured in control solution and in the presence of the toxin at the indicated concentrations, respectively. The dose-response curve was fit with a Hill equation with the only two free parameters being the dissociation constant (K_d) and the Hill coefficient (n). The resulting K_d values and Hill coefficients were the following in the case of Css20: K_d = 1.26 nM, n = 1.17 for Kv1.2 and K_d = 7.21 nM, n = 0.90 for Kv1.3; and in the case of Tst26: K_d = 1.9 nM, n = 0.86 for Kv1.2 and K_d = 10.7 nM, n = 0.72 for Kv1.3. The values of the Hill coefficient indicate that the interaction of the Css20 peptide with the potassium channel pore follows the general blocking scheme of pore blocking toxins: one peptide binds per channel. But values of Hill coefficients for Tst26 seem to be significantly different from one (n = 0.72).

4.3 Influence of Tst26 on Kv1.2 and Kv1.3 channels

Although most known toxin blockers of K^+ channels employ a simple pore blocking mechanism to prevent the permeation of K^+ ions through the pore there are also toxins that bind at or near the voltage sensing regions of the channel and interfere with its gating. These toxins are able to shift the voltage dependence of steady-state activation to depolarized potentials and thus reduce the potassium current. In order to rule out the latter possibility we determined the characteristic steady-state parameters of the voltage dependent activation and inactivation of Kv1.2 and Kv1.3 channels in the absence and the presence of Tst26. First the current-voltage (I-V) function of the

channels was constructed using a series of depolarizing pulses ranging from -70 to +40 mV in 10 mV increments and measuring the peak whole-cell current during each pulse. The voltage dependence of the whole-cell conductance was calculated from the I-V function and the values were normalized to the maximum conductance value. The data were fit with Boltzmann-functions and the comparison of the fit parameters revealed no effect of the toxin on the voltage dependence of steady-state activation of either channel (Kv1.2: control: $V_{1/2}$ = −26.9 mV, and k = 5.2 mV; and 2 nM Tst26: $V_{1/2}$ = −23.7 mV, and k = 5.1 mV; Kv1.3: control: $V_{1/2}$ = −41.2 mV, and k = 7.9 mV; and 10 nM Tst26: $V_{1/2}$ = −40.1 mV, and k = 8.1 mV). Likewise, the voltage dependence of steady-state inactivation for both channels was unaffected by the presence of the toxin (Kv1.2: control: $V_{1/2}$ = −37.3 mV, and k = −5.0 mV; and 2 nM Tst26: $V_{1/2}$ = −44.5 mV, and k = −6.7 mV; Kv1.3: control: $V_{1/2}$ = −50.8 mV, and k = −6.7 mV; and 10 nM Tst26: $V_{1/2}$ = −53.7 mV, and k = −5.6 mV). In contrast to Kv1.2, Kv1.3 channels inactivate significantly during prolonged depolarizations with a time constant of about 200 ms. We tested if the inactivation kinetics of Kv1.3 channels was influenced by the toxin. Time constants of the exponential functions fit to the inactivating part of the current traces were compared in the absence and presence of Tst26. The control value was determined as the average of the time constants measured before toxin application and following toxin wash-out to correct for the inherent acceleration of inactivation of Kv1.3 channels with time. In the presence of the toxin Kv1.3 inactivation was slower than in control conditions; 10 nM Tst26 increased the time constant from 205 ± 33 to 342 ± 37 ms (n = 6, $p < 0.001$). The slowing of the inactivation kinetics in the presence of Tst26 was accompanied by an increase in the steady-state current at the end of the depolarizing, as measured by the ratio of the steady-state current and the peak current (control: 0.021 ± 0.006, 10 nM Tst26 0.042 ± 0.016, n=4, p=0.04).

5. DISCUSSION

Toxins purified from the venom of various species that are highly specific blockers of the Kv1.3 potassium channel have gained much interest in recent years due to the prospect of achieving selective immunosuppression by inhibiting the proliferation of certain lymphocyte subsets. To date no such well defined pharmacological target is known for high affinity blockers of the Kv1.2-channel, since this subtype is widespread in the central nervous system and forms heterotetramer channels with other members of the Shaker family that are likely to have different pharmacological properties from the homotetrameric channels. Thus, a toxin with similar affinities for these two channels, such as Css20 and Tst26, is not an ideal candidate as a potential therapeutic drug. However, there are substantial differences between the inhibitory potencies of Css20 and Tst26 on Kv1.2 and Kv1.3, compared to Kv1.1 and Kv1.4 (well over 100-fold). These 4 channels are closely related with only 9 non-conserved positions over 38 residues which define the interacting surface. Moreover, the blocking kinetics of Kv1.2 (slow) and Kv1.3 (fast) are also significantly different; therefore structure–function studies with such toxins can pinpoint critical residues on channel surfaces and in toxins that determine the affinity of binding and selectivity of toxins for Kv1.2- or Kv1.3-channels. This information then can aid the design of toxins more selective for a particular channel type. Additionally, the analysis reported here opens the field for experiments aiming at expressing specific Css20 and Tst26 mutants, in order to obtain more selective variants of these toxins, with possible preference for one versus the other channels modeled here.

Comparison of the primary sequence of the members of the a-KTx 2 subfamily reveals several highly conserved residues, such as I3, K6, Q12, P16, K18, G22, N31, G32, K33, K28, K35, and Y37. Two of these residues, a critically positioned lysine (K28 in Css20; K27 in Tst26) and an aromatic residue 9 positions downstream (Y37 in Css20; Y36 in Tst26), have been referred to as the ‘‘functional because this pair is found in many high affinity K^+ channel blocking scorpion toxins, not only in members of the α-KTx2 subfamily. The ‘‘functional dyad’’ seems to be critical for the high affinity block of Kv1.2. Mutation of the dyad residues to alanines reduced the affinity of the toxin Pi1 (α-KTx6.1) for Kv1.2 by several orders of magnitude and the two corresponding residues in maurotoxin (MTX (α-KTx6.2), K23

and Y32) also proved essential for high affinity binding to Kv1.2. The requirement for the dyad is not as straightforward for blocking Kv1.3. Despite the presence of the dyad MTX blocks hKv1.3 very poorly, but the H399T mutation at the bottom of the outer vestibule renders it as sensitive to MTX block as Kv1.2 is. The dyad residue Y32 of MTX was found to interact with residue T399 of mutant hKv1.3, so the interaction between H399 in wild type Kv1.3 and the dyad tyrosine in the toxin seems to interfere with the binding. This must be the case for Css20 as well, as our docking simulations indicate interactions between Y37 of the toxin and H399 on two subunits of hKv1.3. Although there are several toxins bearing a tyrosine at the aromatic dyad position, which block Kv1.3 in the nanomolar range, such as noxiustoxin, hongotoxin-1 and Css20, the selectivity for Kv1.3 seems to benefit from the replacement of this tyrosine by other residues. The most effective scorpion toxin blockers of Kv1.3 have a residue at the ‘‘aromatic dyad position’’ different from tyrosine such as phenylalanine (Pi2, Pi3, anuroctoxin; α-KTx7.1, 7.2 and 6.12, respectively), threonine (kaliotoxin, OSK1; α-KTx3.1 and 3.7, respectively) or asparagine (HsTx1 α-KTx 6.3), whereas toxins favoring Kv1.2 over Kv1.3 all have a tyrosine at this position [MTX, Pi1, CoTX1 (α-KTx10.1), Pi4 (α-KTx6.4). Thus, while the critical lysine protruding into the channel pore seems essential for the block of both channels, the tyrosine embodying the other half of the dyad apparently steers selectivity toward Kv1.2. In agreement with this, docking results predict several contacts between the residues Y37 and P38 of the toxin and various residues of both channels. This suggests that steric constraints at the ‘‘aromatic dyad residue’’ position are very important determinants of high affinity binding.

The significance of the different toxin regions in channel recognition was demonstrated using a chimeric toxin constructed from the α-helical N-terminal region of the Kv1.2-specific MTX and the C-terminal region of the Kv1.3-specific HsTx1. The study showed that the replacement of the C-terminal half of MTX, which includes the β-hairpin region, with that of HsTx1 completely abolished high affinity block of Kv1.2 indicating the relevance of this segment in the selectivity between Kv1.2 and Kv1.3. Closer examination of this region reveals differences between Kv1.2- and Kv1.3-specific toxins in the number of residues with basic and acidic side chains. Toxins with higher affinity for Kv1.3 tend to have a higher net charge in general, but more importantly, a higher net charge of the C-terminal half correlates well with higher selectivity for Kv1.3 over Kv1.2. Css20 fits this pattern having only

three basic residues (the dyad and two other lysines) in the C-terminal segment and higher affinity for Kv1.2. Furthermore, docking of Css20 to hKv1.2 and hKv1.3 predicts one hydrogen bond and several hydrophobic contacts of these non-dyad lysines (K33 and K35) with hKv1.2, but none with hKv1.3.

In addition, the residue two positions downstream of the critical lysine, which must be very close to the channel surface considering the protrusion of the lysine side chain into the selectivity filter, is an isoleucine in the high affinity Kv1.2 blockers, while it is a methionine in the Kv1.3-specific toxins. This isoleucine was identified as a key residue in the interaction of both CoTx1 and Pi4 with Kv1.2. The residue at the equivalent position in Css20 is an isoleucine (I30) matching the pattern of toxins selective for Kv1.2, and our docking results confirm that this residue is indeed closer to the bottom of the vestibule of hKv1.2 (ca. 2Å) than that of hKv1.3 (ca. 5Å) resulting in better contacts.

The N-terminal segments of the toxins containing the α-helical regions show no such obvious differences that could account for preferential binding to either of the channels, but the MTX-HsTx1 chimera experiments clearly demonstrate the crucial role of this region in binding as well. The replacement of the N-terminal half of HsTx1 with that of MTX reduced the affinity of the toxin for Kv1.3 almost 400-fold. Three residues in this region of OSK1 were changed for residues found in kaliotoxin (OSK1-12, 16, 20, mutated residues), a highly selective blocker of Kv1.3, which resulted in an almost 10-fold increased selectivity for Kv1.3. The mutation K20D in OSK-1 alone resulted in an almost sevenfold improvement in Kv1.3 selectivity, and the equivalent residue, R14 in CoTx1 was found to interact with Kv1.2, suggesting that a basic residue at this position favors binding to Kv1.2 over Kv1.3. R19 of Pi4 is also an influential residue in binding to Kv1.2 and toxins specific for this channel bear an arginine or a glutamine at this position while toxins with lower affinity for Kv1.2 have uncharged or acidic residues with shorter side chains. In this respect Css20 differs from the high affinity Kv1.2 blocking toxins having two alanines at the equivalent positions (residues 19 and 20). Other residues at the N-terminal region of Css20 are predicted to interact specifically with non-conserved residues at the turret, amongst these: S9 makes several contacts with the channel's residue at position Q357A for hKv1.2 and G375A for hKv1.3 whereas residue K18 of the toxin binds with residues in position E355 of subunit D of the hKv1.2, and T373 of subunit D of

hKv1.3. Based on the criteria above, the low net charge of the C-terminal region, the presence of the dyad tyrosine (Y37), and the isoleucine at position 30 predict the higher affinity of Css20 for Kv1.2, whereas the alanines at positions 19 and 20 (alongside with the analogous interacting pairs between other toxin's N-terminal and channels' turret residues) probably work against it, resulting in a moderate selectivity for Kv1.2 over Kv1.3.

There is an extra lysine in the C-terminal region of Tst26 as compared to Css20 and maurotoxin, and methionine instead of isoleucine at position 29. These would steer the selectivity of Tst26 toward Kv1.3, while Q19 would favor binding to Kv1.2. Overall both Css20 and Tst26 toxins show slight (5-fold) preference for Kv1.2. Additionally, they are likely to follow similar docking strategies to both channels since the association and dissociation rates are also comparable.

Both the development of steady-state block by Css20 and recovery from block were complete in 30 s for Kv1.3, while block development was about 10-times slower and recovery at least 30-times slower for Kv1.2. The striking differences in the rates of toxin association and dissociation to Kv1.2 and Kv1.3 indicate dissimilar modes of interaction with these channels in spite of the similar affinities. The fast association rate to Kv1.3 implies fast orientation at the early steps of docking, probably via through-space electrostatic interactions, but a number of unfavorable close contact interactions are likely to be the reason for the equally fast dissociation. Docking results are in complete agreement with this scenario; most of the interacting residues of hKv1.3 are those of the selectivity filter, which make contacts mainly with the toxin's plugging residue K28. On the other hand, toxin orientation during docking to Kv1.2 seems less aided by through-space electrostatics, but the contact surface of Css20 better suits Kv1.2, with an extensive hydrogen bonding network – as predicted by the docking – between channel's turret residues and axially located residues of the toxins, leading to slower dissociation and an overall higher affinity.

Development of Kv1.2 block by Tst26 carrying one extra positive charge was somewhat faster than that by Css20, presumably due to through-space electrostatic interactions between the receptor site in the extracellular entrance of the channels and the toxin. However, dissociation was also faster, taking about 6 minutes for full recovery during wash-out, which may be attributed to some less favorable close-contact interactions between Q19 and M29 in Tst26 and their respective partner

residues on the channel surface than those of the equivalent residues A19 and I30 in Css20.

An unusual feature of Tst26 is the slowing of the inactivation rate of Kv1.3 channels. This phenomenon could be explained by the "foot-in-the-door" model described earlier for tetraethylammonium, which states that the presence of an appropriate blocker impedes the conformational changes required for slow inactivation. The conformation of the selectivity filter and its K^+ occupancy controls slow inactivation, both of which can be influenced by a bound toxin. The side chain of the critical lysine of scorpion toxins protrudes into the selectivity filter of the channel and toxin binding induces conformational changes in the channel. Furthermore, the bound toxin may trap K^+ residing in the selectivity filter thereby preventing the conformational changes leading to slow inactivation. For most toxins the residency time on the channel is much longer than the inactivation process, thus only a negligible number of channels is unblocked during the depolarizing pulse and contributes to the whole-cell current. However, Tst26 block of Kv1.3 develops and is fully reversed within 15 seconds, meaning that the time constants of association and dissociation are in the order of a few seconds. Unfortunately, the blocking kinetics of Tst26 cannot be determined with better time resolution due to an inherent property of Kv1.3 channels called cumulative inactivation that develops in response to a series of short depolarizations, and thus, limits the frequency of peak current determinations. Nevertheless, toxin association and dissociation time constants are comparable to the duration of the inactivation process (1-1.5 sec), so multiple association and dissociation events may occur during the depolarizing pulse. Thus, channels whose inactivation is hindered by the interaction with Tst26 can contribute to the whole-cell current resulting in slower macroscopic inactivation kinetics. This phenomenon is also apparent in the increased steady-state current to peak current ratio in the presence of the toxin during long depolarizing pulses. Since the steady-state current level is determined by the relative rates of entering and exiting the inactivated state, the slowing of the forward rate will result in a higher fraction of non-inactivated channels, thus, a higher steady-state current level, consistent with our findings.

6. SUMMARY

We identified and characterized new peptide toxins: the Css20 from the venom of the scorpion *Centruroides suffusus suffusus* and the Tst26 from the venom of the scorpion *Tityus stigmurus*. These toxins were shown to block preferentially the currents of the voltage-dependent K^+ channels Kv1.2 and Kv1.3 with high affinity (Css20: K_d = 1.3 nM and 7.2 nM, Tst26: K_d = 1.9 nM and 10.7 nM, respectively). These toxins did not affect several other voltage-dependent (mKv1.1, hKv1.4, hKv1.5, hBKCa, hERG together with rKv2.1 for Css20) and calcium-activated (hIKCa1) potassium channels as well as cardiac sodium channel (hNav1.5).

The striking differences in the rates of toxin association and dissociation to Kv1.2 and Kv1.3 indicate dissimilar modes of interaction with these channels in spite of the similar affinities.

In the case of Tst26 the voltage dependence of steady-state activation and inactivation of Kv1.2 and Kv1.3 channels was unaffected by Tst26. But because of fast association and dissociation kinetics Tst26 decreased the inactivation of Kv1.3 channel.

Css20 is composed of 38 and Tst26 is composed 37 amino acid residues and tightly folded through three disulfide bridges, similar to other K^+ channel blocking peptides purified from scorpion venoms. Both contain the "essential dyad" for K^+ channel recognition comprised of a lysine at position 28 (K28) and an aromatic residue (here tyrosine) at position 37 (Y37) for Css20. These residues are K27 and Y36 for Tst26. Based on the primary structures, the systematic nomenclatures proposed for these peptides are α-KTx2.13 for Css20 and α-KTx4.6 for Tst26.

The observations from sequence comparisons, mutant cycle analyses and models of toxin docking can enable us to improve toxin selectivity for either of the channels. For instance, disrupting Kv1.2-specific interactions of Css20 (Q11 and K33 with E355 in different subunits), could increase Kv1.3 selectivity. Such an approach, successful for other scorpion toxins, would allow targeting appropriate cells and physiological/pathophysiological functions. Further studies are required to explore this scenario, which can be guided by the analysis presented in this study.

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