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Molecular approaches for structural characterization of a new potassium channel blocker from *Tityus stigmurus* venom: cDNA cloning, homology modeling, dynamic simulations and docking

Diego Dantas Almeida [a,b], Taffarel Melo Torres [b], Euzébio Guimarães Barbosa [c], João Paulo Matos Santos Lima [b], Matheus de Freitas Fernandes-Pedrosa [a,b,*]

[a] Laboratório de Tecnologia e Biotecnologia Farmacêutica, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil
[b] Programa de Pós-Graduação em Bioquímica, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil
[c] Laboratório de Química Farmacêutica, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil

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ABSTRACT

Potassium channels are involved in the maintenance of resting membrane potential, control of cardiac and neuronal excitability, neurotransmitters release, muscle contractility and hormone secretion. The *Tityus stigmurus* scorpion is widely distributed in Northeastern Brazil and known to cause severe human envenomations, inducing pain, hypoesthesia, edema, erythema, paresthesia, headaches and vomiting. Most potassium channel blocking peptides that have been purified from scorpion venoms contain 30–40 amino acids with three or four disulfide bridges. These peptides belong to α-KTx subfamily. On the other hand, the β-KTx subfamily is poorly characterized, though it is very representative in some scorpion venoms. A transcriptomic approach of *T. stigmurus* scorpions developed by our group revealed the repertoire of possible molecules present in the venom, including many toxins of the β-KTx subfamily. One of the ESTs found, named TSTI0003C has a cDNA sequence of 538 bp codifying a mature protein with 47 amino acid residues, corresponding to 5299 Da. This β-KTx peptide is a new member of the BmTXKβ-related toxins, and was here named TstKMK. The three-dimensional structure of this potassium channel toxin of the *T. stigmurus* scorpion was obtained by computational modeling and refined by molecular dynamic simulations. Furthermore, we have made docking simulations using a Shaker kV-1.2 potassium channel from rats as receptor model and proposed which amino acid residues and interactions could be involved in its blockade.

1. Introduction

Through a long evolutionary time, scorpions developed venomous glands with a variety of biologically active molecules [1]. The best-known among these are the neurotoxin peptides that affect ion channels. Such peptides are useful for predation and are involved in numerous clinical concerns [2].

The scorpion venom neurotoxins are composed of two major polypeptide populations. One consists of several long-chain toxins affecting Na^+ channels, and the other one includes short-chain toxins affecting K^+ or Cl^- channels on both excited and non-excited cell membranes [3–5]. These toxins are composed of an α-helix connected to a double- or triple-stranded β-sheet by highly conserved disulfide bridges (CSαβ-motif). Most specific K^+ channels scorpion toxins (KTx) are short-chain peptides comprising 23–42 amino acid residues cross-linked by 3–4 disulfide bridges [5]. These are classified as α-KTx toxins, most of which block voltage-gated K^+ channels (kV) by a dyad motif directly interacting with the channel pore [6]. However, there are reports of some toxins that lack the dyad, but still maintain a high-affinity binding capacity to kV channels [7].

Another group of related potassium channel toxins are named β-KTx, which comprises 61–75 amino acid residues. This group is represented by few peptides from Buthidae, Caraboctonidae and Scorpioninae families [5] and can be subdivided into three classes [8]: (1) TsTXKβ (TstβKTx)-related peptides, characterized as a kV-channel blocker, (2) BmTXKβ-related peptides (a blocker of transient outward K^+ current (I_{to}) in rabbit atrial myocytes) [9,10] and (3) Scorpine-related peptides, a group of antimicrobial defensins [11]. These peptides contain six cysteines forming three disulfide bridges, and present two structural domains: a putative α-helical N-terminus and a Cys-rich C-terminus, with the consensus signature of CSαβ-motif, which is involved in the potassium

* Corresponding author. Address: Universidade Federal do Rio Grande do Norte, Av. Gal. Cordeiro de Farias, s/n, CEP 59012-570, Natal, RN, Brazil. Fax: +55 84 3342 9804.
E-mail address: mpedrosa@ufrnet.br (M. de Freitas Fernandes-Pedrosa).

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channel blocking effect [12,13]. Some of the β-KTx members also exhibit antimicrobial and cytolytic activity [8].

The *Tityus stigmurus* (Thorell, 1876) belongs to the Buthidae family, which comprises all scorpions harmful to human worldwide, being the *T. stigmurus* one of the most medical important scorpions in Brazil. Since the isolation of the first scorpion venom peptide in the 1980s [14], a large diversity of toxins that block potassium channels has been characterized [15] or predicted [16–20] in several scorpion genus, including *Tityus*. Nevertheless, little is known about the *T. stigmurus* venom [21]. As a diverse group of integral membrane proteins, potassium channels can be found in many types of tissues as muscles, neurons, pancreatic β-cells, lymphocytes and fat cells [22], either controlling electrical excitability or playing important roles in signaling pathways [23]. Scorpion neurotoxins are able to block K^+ channels through an authentic pore occlusion mechanism, hence these molecules are excellent ligand models for studying K^+ channel function and structure [24].

The mKv1.2 is a voltage-gated potassium channel belonging to the Shaker superfamily. It shows a tetrameric geometry with identical subunits [25], each containing six transmembrane α-helical segments S1–S6 and a membrane-reentering p-loop (P). The channel central pore is formed by S5–S6 linkers and the p-loop of each monomer. The K^+ conduction pathway contains the TVGYG sequence, a widely conserved K^+ channel signature, which has a critical role in potassium selectivity [22,24]. The discovery of new K^+-channel toxins may provide valuable tools for exploring the structure–function of ion channels. Moreover, K^+-channel toxins are also useful in drug research and discovery because they can be used as pharmacological tools for understanding of many physiological processes and uncovering potential therapeutic targets [26–28].

Our group has studied the repertoire of possible molecules present in the venom of *T. stigmurus* scorpions using a transcriptomic approach. The data set revealed some classes of toxins contained in the *T. stigmurus* venom, including potassium channel toxins, representing approximately 14% of the total transcripts [21]. In the present work, we report the successful cloning of a transcript encoding an unidentified potassium channel blocker from *T. stigmurus*, and describe its molecular structural basis and evolutionary relationships using a molecular modeling approach and phylogenetic analysis, respectively. The peptide tridimensional structure was proposed and further validated by dynamic and docking simulations.

2. Materials and methods

2.1. cDNA cloning and bioinformatic analyses

A full-length cDNA library was prepared using the *In-Fusion™ SMARTer™ cDNA Library Construction Kit* (CLONTECH Lab., Palo Alto, CA) as described elsewhere [21]. The complete nucleotide sequence TSTI0003C was analyzed by ORF-Finder [http://www.ncbi.nlm.nih.gov/projects/gorf/]. The signal peptide was predicted with the SignalP 3.0 program, using both neural networks (NN) and hidden Markov models (HMM). A secretory protein was considered when both methods showed a signal peptide according to their default parameters (mean $S > 0.048$ and mean D score $0.43 >$ in NN and signal peptide probability > 0.5 in HMM). The pro-peptide sequence was identified following the rational from Diego-Garcia et al. [9], and subsequently removed from the final FASTA sequence [21].

2.2. Phylogenetic analysis

The TSTI0003C amino acid FASTA sequence was used in a BLAST search against the nr database, including sequences of "TsTXKβ (TstβKTx)-related peptides", "BmTXKβ-related peptides", "Scorpine-related peptides" and "Defensins" from arthropods as outgroup (Table 2, Supplementary material). The resulting 30 sequences were then submitted to an alignment using the programs MAFFT [29] at the amino acid level using the L-INS-i algorithm, and MUSCLE [30] with custom alignment parameters. A best-fit adjustment of the amino acids substitution model was performed using the ProtTest tool [31]. The dendograms were calculated based on a Bayesian analysis, using the packages MrBayes 3.1.2 [32] and BEAST [33]. The Bayesian inferences were conducted using 4 independent runs, each one with four simultaneous chains with fixed WAG model [34], allowing gamma distributed rates among sites. Each Markov Chain was initiated with a random tree and ran for 10^6 generations, sampled every 100 generations, and a consensus tree was estimated by using a *burn-in* of 1,000,000 trees. The convergence of the simultaneous runs was assessed using the Tracer tool [35], in order to evaluate the statistic support and robustness of the Bayesian analysis.

2.3. Toxin modeling

The search for suitable templates for the *T. stigmurus* toxin homology modeling was performed with the MODELLER 9.10v suite [36], using a formatted PDB sequences database (database version 05/24/2011). The search considered the following parameters: BLOSUM62 amino acid substitution matrix, gap open penalty of −500, gap extension penalty of −50, and the *e*-value threshold of 5. From the chosen templates (Table 1, Supplementary material), 500 theoretical models were generated for TSTI0003C.

The toxin was also modeled in Phyre2 Web Server [37] and I-TASSER [38], the best CASP9 [39] protein predictors. Ten repetitions rounds and the intensive mode were applied in Phyre2. For I-TASSER we used 20 templates chosen automatically and 14 simulations to generate 10 models for the toxin. The DOPE Scores, Ramachandran plots and the Cβ deviation parameters were calculated for all models. The first one was determined by MODELLER and the latter two were calculated using the *MolProbity* program. The models were analyzed and sorted by scores values, and the pdb files were visualized using USFC Chimera software. The top three models, one for each approach, were submitted to a molecular dynamics analysis.

2.4. Homology models refinement

Molecular dynamics simulations were performed to optimize the obtained homology models. All simulations were performed using explicit water (TIP3P), using the GROMACS [40] Simulation package and AMBER99SB-ILDN [41] force field. The protonation state of the proteins residues was determined at pH 7.0 by PROPKA [42] web server. Counterions were added to neutralize the system. The molecular dynamics simulations were performed at constant temperature and pressure in a periodic truncated triclinic box. The simulation time step was of 2 fs. The minimum distance between any atom of the protein and the box wall was 1.2 nm. Coulomb and van der Waals interactions within a shorter-range cutoff of 1.0 nm were computed every time step. To minimize the effects of truncating the electrostatic interactions beyond the 1.2 nm long-range cutoff, the Particle Mesh Ewald was employed. Covalent bonds in the protein were constrained using the LINCS algorithm [43]. Prior to molecular dynamics simulation the peptide had its geometry energy minimized using a steepest descent algorithm, followed by conjugate gradient algorithms. A 10-ps protein position restrained molecular dynamics was performed at 300 K to gently relax the water molecules and side chains. Unrestrained molecular dynamics were then performed at 310 K for at least 50 ns to assess the stabilization of the density of the box. During

the simulations the temperature and the pressure were maintained at 310 K and 1 bar by rescaling velocities and using isotropic pressure bath with Parrinello–Rahman barostat. The relaxation times were of 0.1 and 0.5 ps, respectively.

2.5. Molecular protein–protein docking

The refined molecular dynamics and homology model structures of the venom peptide were submitted to the Cluspro server [44] to be docked to Kv1.2 protein structure (PDB code: 3LUT) [25]. The Cluspro server is based on a Fast Fourier Transform correlation approach that is able to evaluate billions of docked conformations. The server returns the top models based on energy and cluster size. The VdW+Electrostatics ClusPro score provided possible structures to explain the mechanism of action for the venom peptide at molecular level. The lowest ClusPro score was chosen to be analyzed. The molecular graphics were created using the USFC Chimera software [45].

3. Results and discussion

TSTI0003C presents a cDNA of 538 bp codifying a mature protein with 47 amino acid residues [GenBank: JK483711] corresponding to an estimated isoelectric point and size of 8.60 and 5299 Da, respectively. The protein sequence shows a 25 amino acids long signal peptide, followed by a pro-sequence with 18 amino acids, revealing a total of 43 amino acids preceding the mature protein. The mature potassium channel toxin includes the motifs CXXXC and CXC located in 24–28 and 42–44 amino acids positions, respectively. The TSTI0003C product shares a high sequence similarity to other *Tityus* toxins [TtrKIK (Q0GY4), TcoKIK (Q0GY42) and TdiKIK (Q0GY43)], but unlike these, it starts with the "KMK" sequence instead of "KIK", and thus named TstKMK (Fig. 1).

Structurally, β-KTxs are polypeptides with 45–68 amino acid residues, including 6 cysteines and the two structural domains [8]. The alignment of TSTI0003C (TstKMK) and sequences of "TsTXKβ (TstβKTx)-related peptides", "BmTXKβ-related peptides", "Scorpine-related peptides" and "Defensins"indicated that β-Ktx from *T. stigmurus* scorpion presents high similarity with β-Ktx isolated from other scorpions. Furthermore, this structural analysis revealed the presence of the two structural motifs CXXXC and CXC (Fig. 1A, Supplementary material). We also performed studies to find out the evolutionary relationships among scorpion toxin sequences as demonstrated by the phylogeny tree. The phylogenetic tree demonstrated that the new β-Ktx of the *T. stigmurus* scorpion described in this work belongs to "BmTXKβ-related peptides", showing high similarity with *Tityus. costatus* and *Tityus. trivittatus* "BmTXKβ" (Fig. 2B, Supplementary material). It can also be concluded that the three toxin subfamilies have a common ancestral protein, and are paraphyletic to the defensins clade (Def). The "BmTXKβ-related peptides" diverged first, and the ("TsTXKβ (TstβKTx)-related peptides" and "Scorpine-related peptides" are probably more recent. These results are strongly supported by high PP values (PP > 0.90). Based on the data above, TSTI0003C (TstKMK) should be a novel putative β-Ktx and belongs to the subfamily "BmTXKβ".

The α-KTX peptides are known to be authentic pore blockers. A good body of evidence indicates that all these peptides bind in the outer vestibule of the channel and block ion conduction by physically occluding the pore, without affecting the kinetics of channel gating. Binding of the peptides occurs through a reversible, bimolecular reaction, which is governed by electrostatic interactions

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01   ggactctcttctctgtcgaattcgag atg gtg gcc acg aat cgt tgc   47
01                              M   V   A   T   N   R   C    07
48   tgt gtc ttc gca ctg ttg ttt gcg ctg ctg ctg gtt cac tcc   89
08    C   V   F   A   L   L   F   A   L   L   L   V   H   S   21
90   ctg acg gag gcg gga aaa gga aaa gaa atc tta gga aaa atc   131
22    L   T   E   A   G   K   I   K   E   K   I   G   K   G   35
132  aag gag aaa ata atc gaa gcg aaa gac aag atg aag gct gga   173
36    K   E   I   L   I   E   A   K   D   K   M   K   A   G   50
174  tgg gaa agg ttg acg tca cag tcg gag tac gcc tgt ccc gcc   215
51    W   E   R   L   T   S   Q   S   E   Y   A   C   P   A   65
216  att gat aag ttc tgc gag gac cat tgc gcc gct aag aaa gcc   257
66    I   D   K   F   C   E   D   H   C   A   A   K   K   A   79
258  gtc gga aaa tgc gac gat ttc aag tgc aag tgc atc aaa ttg   299
80    V   G   K   C   D   D   F   K   C   K   C   I   K   L   93
300  taa tctctgtaaatcctcttctttacgtcaattaaaacaatattcgtggacaagc 355
94    *
356  aagtctcgtctggttaattcttcgtaaatcgtaatcacgattcgttccatacgatt 411
412  gcctttaagaagggatcggttgacagtttatttttatcctattttatattatttgc 467
468  gaacaattcaaaaaaatatctcagttttatt(aataaa)ttttctgattctgaaat 523
524  taanaaaaaaaaa                                           535
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Fig. 1. Full-length cDNA and putative amino acid sequences of potassium channel toxin (TSTI0003C). The protein sequence was predicted using the TRANSLATE tool of ORF-Finder [http://www.ncbi.nlm.nih.gov/projects/gorf/]. The putative signal peptide is underlined, the pro-sequence is underlined with a dotted line and the mature protein is in bold italics. The asterisk denotes the stop codon, the polyadenylation signal is between parentheses. The conserved motifs are in boxes.

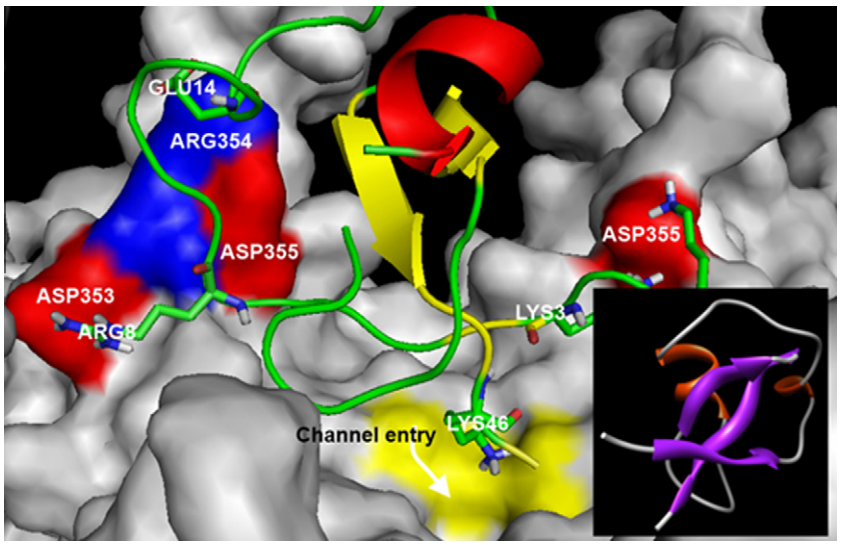

Fig. 2. Molecular docking of the homology model after the molecular dynamics simulations.

between pore residues in the channel and the positively charged residues in the peptide. Positive lysines are critical residues in venom peptide [24]. In the other hand, β-KTx are poorly characterized [8], mainly because the cytolytic effect produced by some of these toxins turns the potassium channel blocking activity difficult to assess.

In most high affinity kV channel blocking scorpion peptides, a strategically positioned lysine and an aromatic residue nine positions downstream form the 'functional dyad' [6]. Therefore, it was hypothesized by Diego Garcia et al. [8] that BmTXKb-related peptides might bind to K^+ channels in an analogous fashion to that of α-KTx, because they have a conserved lysine in a position homologous to those of most α-KTx, although some BmTXKb-related toxins do not have it [13].

Hence, the best scored homology model was docked to a Shaker K^+-channel kV-1.2 model (3LUT). The Lys3 lodged physically close to a potassium binding site in the ion conduction pore (data not shown). But it was expected that Lys36 blocked the channel. Subsequently, a manual docking of Lys36 was performed to verify this and we found out that some prohibitive atomic overlays impaired the homology model to dock as expected. To solve this conundrum a molecular dynamics simulation to relax the homology models and provide a structure able to dock as expected was carried out. The molecular dynamics simulations distorted the homology model mainly on the α-helix. The β-strands persisted but twisted due to internal residues adaptations (Fig. 2). Moreover, when such model was submitted to docking experiments, the Lys36 did not dock as expected, but instead Lys46 poorly docked into the channel. It was thought that the N-terminal domain was hindering the access of Lys36 to the channel pore, so a homology model without it was built, leaving only the Csαβ-motif available. The new pruned model docked Lys46 again persisting the interaction in an obstructive fashion with the pore (data not showed).

The requirement of the 'functional dyad' (including Lys36 in this case) for potassium channels blockade by scorpion toxins could be arbitrary. Scorpion toxins completely lacking this dyad, with effective potassium channel blocking activity was also described [7]. In addition, studies carried out by Mouhat et al. [46], about the role of the 'functional dyad' from *Pandinus imperator* Pi1 potassium toxin, proved that the knockout of such residue does not eliminate the toxin ability to block such channels. Moreover, based on the Bayesian analysis results, since the toxin subfamily

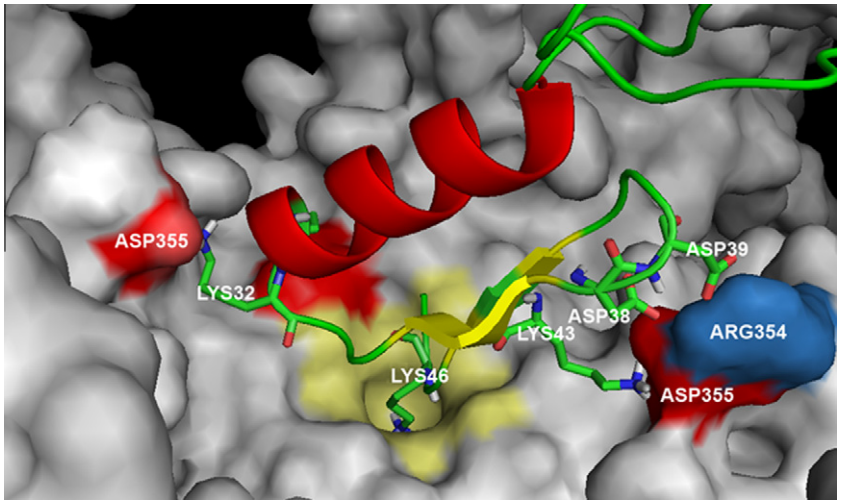

Fig. 3. Binding mode of a complete model of TstKMK. The N-terminus is presented as a random coil.

BmTXKβ is apparently more ancient, it may show a more generalist action mechanism, that can inactivate diverse kV channels, providing more than one docking site possibility. So we propose that Lys46 is likely to be an important residue in the inhibition mechanism of TstKMK for this channel type.

The position of the N-terminal moiety was investigated using its alternative positioning provided by the Phyre2 server, which attributed α-helix as preferred secondary structure. Two Phyre2 models were further optimized by means of molecular dynamic simulations and provided suitable for Lys46 to dock in an inhibitory conformation and the adjacent residues were complementarities to the binding site. The structure is presented in Fig. 3.

During the molecular dynamics simulation it was verified that one of the Phyre2 models was more stable, presenting the shortest radius of gyration. The N-terminus undertook a variety of shapes and positioning, but the residue of the peptide chain remained almost constantly in one α-helix and two antiparallel β-strands motif (Video Supporting Information). Our hypotheses is that the N-terminus adopts a conformation able to optimize the interaction between the toxin and the channel when atop of the binding pocket, but may assume different conformations while free in the bulk of the secreted venom. The best scored I-TASSER homology model may be a conformation accessible to this toxin but it might be able to undertake a major conformational change in order to bind to the Kv1.2 channel. This hypothesis is supported by data from another β-KTx of the *Mesobuthus eupeus* scorpion named MeuTXKβ1, whose structure is very affected by the medium. This toxin changes its α-helical content (especially in the N-terminus) according to an aqueous or membrane mimicking environment [12].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.044.

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