

BmTx3, a scorpion toxin with two putative functional faces separately active on A-type K⁺ and HERG currents

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A novel HERG channel blocker was isolated from the venom of the scorpion *Buthus martensi* Karsch, sequenced and characterized at the pharmacological level after chemical synthesis. According to the determined amino acid sequence, the cDNA and genomic genes were then cloned. The genomic gene consists of two exons interrupted by an intron of 65 bp at position –6 upstream from the mature toxin. The protein sequence of this toxin was completely identical with that of a known A-type K⁺ current blocker BmTx3, belonging to scorpion α -KTx subfamily 15. Thus BmTx3 is the first reported α -KTx peptide also showing HERG-blocking activity, like γ -KTx peptides. Moreover, different from classical α -KTx peptides, such as charybdotoxin, BmTx3 cannot block *Shaker*-type K⁺ channels. Phylogenetic tree analysis reveals that this toxin takes an inter-

mediate position between classical α -KTx and γ -KTx toxins. From a structural point of view, we propose that two separate functional faces might exist on the BmTx3 molecule, responsible for the two different K⁺-current-blocking functions. Face A, composed of Arg¹⁸ and Lys¹⁹ in the α -helix side, might correspond to HERG blocking activity, whereas Face B, containing a putative functional dyad (Lys²⁷ and Tyr³⁶) in the β -sheet side, might correspond to A-type blocking activity. A specific deletion mutant with the disrupted Face B, BmTx3-Y36P37del, loses the A-type current-blocking activity, but keeps a similar HERG-blocking activity, as seen with the wild-type toxin.

Key words: *Buthus martensi* Karsch, gene cloning, phylogenetic tree, potassium channel, structure modelling, scorpion toxin.

INTRODUCTION

Scorpions have survived for more than 350 million years without detectable changes in their anatomies. This distinctive evolutionary phenomenon is due to their efficient production of different neurotoxins with diverse ion-channel-blocking activity [1]. Potassium channels form a very diverse superfamily of more than 80 members that play crucial roles in many different physiological processes [2]. To increase the efficiency of blocking diverse potassium channels and also save energy, scorpions produce neurotoxins that can act on multiple potassium channels. For instance, charybdotoxin (ChTx), the well-studied potassium-channel blocker from the scorpion *Leiurus quinquestriatus hebraeus*, is able to block *Shaker*, Kv1.3, K_{Ca}1.1 and K_{Ca}3.1 channels [3]. Mutagenesis studies on this toxin showed that ChTx used the same functional face located at its C-terminal β -sheet region to block multiple potassium channels [4,5]. This β -sheet functional face is also shared by many other toxins. In this face, a functional dyad composed of lysine and an aromatic residue always play a key role in toxin–channel interaction [6].

In the present study, the venom from the scorpion *Buthus martensi* Karsch (*BmK*) was screened with the goal to characterize a HERG (human *ether-a-go-go*-related gene) channel blocker. A toxin composed of 37 amino acid residues cross-linked by three disulphide bridges was purified. The cDNA and genomic

genes were then cloned and sequenced. The protein sequence is completely identical with the known toxin BmTx3, an A-type K⁺-current blocker with a K_d of 54 nM [7]. BmTx3, which is in fact the same toxin as BmTx3A, has already been classified into the scorpion α -KTx15 subfamily, together with AmmTx3, Aa1 and Aa1 analogues [8–11]. Therefore, BmTx3 is a scorpion toxin capable of blocking two completely different K⁺ currents (A-type and HERG K⁺ currents).

So far, it is still not entirely clear which type of potassium channel is responsible for A-type K⁺ current, at the molecular level. In contrast, the HERG channel has been well studied because of its important role in cardiac arrhythmias. As a product of the human *ether-a-go-go*-related gene, the HERG channel plays a crucial role in the repolarization phase of the cardiac action potential [12]. Mutations on the HERG channel are responsible for the inherited long QT syndrome (LQT2) that may cause syncope and sudden death resulting from arrhythmias and ventricular fibrillation [13]. Unique among the other known voltage-activated K⁺ channels, the HERG channel shows the characteristics of inward rectifiers functionally, due to its extremely fast inactivation process, which is associated with its distinctive long turret region. The long turret region forms an amphipathic α -helix, which plays a central role in the toxin–channel interaction [14,15]. Mutagenesis results of the HERG toxin BeKm-1 from scorpion *Buthus eupeus* showed that the functional face of BeKm-1 shifted

Abbreviations used: *BmK*, *Buthus martensi* Karsch; ChTx, charybdotoxin; GSP, gene-specific primer; HERG, human *ether-a-go-go*-related gene; *I*_{fully activated}, inward current; *I*_t, small outward current; *I*_{tail}, large outward tail current; KTx, K⁺ channel toxin; RACE, rapid amplification of cDNA ends; RP-HPLC, reverse-phase HPLC; sBmTx3, synthetic BmTx3; TFA, trifluoroacetic acid; UTR, untranslated region; V_{1/2}, half maximal potential; V_t, test potential.

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The nucleotide sequences for cDNA and genomic gene of BmTx3 has been deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AF541980 and AY156725 respectively.

from the conserved β -sheet face to the α -helix face. Two basic residues (Lys¹⁸ and Arg²⁰) in this face are crucial for the interaction of the toxin with the amphipathic α -helix of HERG channel [16,17].

Based on the NMR structure of an homologous toxin, TyK α [18], the BmTx3 structure was modelled by the SWISS-MODEL server (<http://www.expasy.org/swissmod/SWISS-MODEL.html>). A putative functional dyad composed of Lys²⁷ and Tyr³⁶ was found in the C-terminal β -sheet region. Furthermore, two basic residues (Arg¹⁸ and Lys¹⁹) were located in the α -helix face corresponding to Lys¹⁸ and Arg²⁰ of BeKm-1. These results imply that BmTx3 might have two different functional faces responsible for different channel-blocking functions. Mutagenesis studies were then performed to verify this idea.

EXPERIMENTAL

Materials

BmK scorpions were collected from the Henan Province, China. The Sephadex G-50, the DEAE-Sephacel column and the Mono-S column were purchased from Amersham Biosciences (Uppsala, Sweden), the semi-preparative reverse-phase HPLC (RP-HPLC) C₁₈ column from Beckman (Fullerton, CA, U.S.A.), the 218TP104 RP-HPLC C₁₈ column from Vydac (Hesperia, CA, U.S.A.). Trifluoroacetic acid (TFA) and acetonitrile (HPLC grade) were from Merck (Darmstadt, Germany). The 3' and 5' RACE (rapid amplification of cDNA ends) kits, the TRIzol[®] reagent and the T4 DNA ligase were purchased from Life Technology (Gaithersburg, MD, U.S.A.), restriction endonucleases and *Taq* DNA polymerase were from MBI (Vilnius, Lithuania) and Sangon (Shanghai, China) respectively. The pGEM-T-Easy vector and the DNA sequencing kit were purchased from Promega (Madison, WI, U.S.A.). The gene-specific primers were chemically synthesized by Sangon Company (Shanghai, China). The T7 RNA polymerase and a cap analogue diguanosine triphosphate were from Promega Benelux b.v. (AJ Leiden, The Netherlands). DNA extraction was performed using the Qiagen extraction kit (Studio City, CA, U.S.A.). The T7 mMESSAGE mMACHINE transcription kit was from Ambion (Austin, TX, U.S.A.). Pyroglutamate aminopeptidase, tricine and collagenase were from Sigma (Belgium). Ethanoic acid, sodium ethanoate, NaCl and other reagents were of analytical grade.

Purification and synthesis of BmTx3

Monitored by the electrophysiological experiments, the purification of the HERG channel blocker from scorpion *BmK* was performed as follows. The venom of the scorpion *BmK* was first fractionated on a Sephadex G-50 column (1.2 cm \times 155 cm), as previously described [19]. Peak 2, containing various neurotoxins, was applied on to a DEAE-Sephacel column (3 cm \times 6.5 cm), equilibrated previously with 20 mM Na₂CO₃/NaHCO₃ buffer, pH 10.5. The breakthrough fraction was pooled and loaded on to a semi-preparative RP-HPLC C₁₈ column (1 cm \times 25 cm), equilibrated with buffer A (0.1% TFA in water) at a flow rate of 2 ml/min. Proteins were eluted by a two-step gradient system: 0–36% buffer B (70% acetonitrile in buffer A) for 36 min and 37–46% buffer B for 15 min. Fraction 3 from the C₁₈ column was applied to a Mono-S column (0.5 cm \times 5 cm), equilibrated with 50 mM ethanoic acid/sodium ethanoate buffer, pH 4.3, at a flow rate of 1 ml/min. Elution was carried out by a linear gradient of 0–1 M NaCl in the ethanoic acid/sodium ethanoate buffer for 40 min. The purified component of the first peak (BmTx3) was then sequenced by Edman degradation on an Applied Biosystems 491 pulsed-liquid-phase sequencer (Foster City, CA, U.S.A.).

According to the determined sequence, BmTx3 and its analogue (BmTx3-YPdel) have been chemically synthesized as previously described [7,20]. The native and synthetic BmTx3 were co-eluted on a 218TP104 C₁₈ RP-HPLC column (4.6 mm \times 250 mm), indicating that the synthetic toxin is identical with the native one.

RACE

Total RNA was extracted from the scorpion venomous gland as previously described [21]. Approx. 5 μ g of total RNA was taken to convert mRNA into cDNA using Superscript II reverse transcriptase. Based on the determined protein sequence, gene-specific primers (GSPs) 1 and 2 were designed and synthesized for the 3' RACE. GSP 1 [5'-AA(T/C)GT(A/T/G/C)AA(A/G)TG-(T/C)CA(A/G)GG-3'] and GSP 2 [5'-AA(A/G)GC(A/T/G/C)-AT(A/C/T)GG(A/T/G/C)GT(A/T/G/C)GC-3'] corresponded to amino acid residues 5–10 (NVKCQG) and amino acid residues 19–24 (KAIGVA) respectively. Using the converted cDNA as a template, the 3' partial gene of BmTx3 was first amplified by PCR with GSP1 and an abridged universal adaptor primer (AUAP). To obtain a more specific product of BmTx3 cDNA, the original PCR product was diluted and amplified further with AUAP and the nested primer GSP2. The amplified product was cloned into pGEM-T-Easy vector for sequencing.

Based on the 3' partial cDNA sequence, the anti-sense primers, GSP3 and GSP4, were designed for the 5' RACE. GSP3 (5'-CGGGTAGCAAACACATC-3') and GSP4 (5'-TCCGTTAATGC-ATTTGCC-3') corresponded to amino acid residues 37–32 (RCVCYP) and amino acid residues 31–26 (GKCING) respectively. The 5' partial sequence of BmTx3 cDNA was obtained as described previously [22].

The full-length cDNA was then obtained by overlapping 3' and 5' partial cDNA sequences, and it has been deposited in GenBank[®] under the accession number AF541980.

Amplification of the genomic gene

The total genomic DNAs were isolated from scorpion tail glands by the methods described previously [21]. A pair of primers GSP5 and GSP6 were designed for amplifying the genomic gene of BmTx3. GSP5 (5'-ATAAAAGCTTTATCAGAAGA-3') corresponded to the 5'-UTR (untranslated region) just upstream of the initial codon ATG, and GSP6 (5'-ATAAATTATCATTACATCG-3') corresponded to the anti-sense sequence of the 3'-UTR just downstream of the stop codon TAG. The amplified genomic gene was cloned into pGEM-T-Easy vector for sequencing. The obtained gene sequence has been deposited in GenBank[®] under the accession number AY156725.

Electrophysiological recording

Injection and expression

Stage V–VI *Xenopus laevis* oocytes were isolated as previously described [23]. The oocytes were defolliculated by treatment with 2 mg/ml collagenase in calcium-free ND-96 solution. Between 2–24 h after defolliculation, oocytes were injected with 50 nl of 1–100 ng/ μ l cRNA using a Drummond micro-injector (Sarasota, FL, U.S.A.). The oocytes were then incubated in ND-96 solution at 18 °C for 1–4 days.

The *in vitro* syntheses of cRNAs encoding Kv1.1, Kv1.2, Kv1.3 and Kir2.1 were performed as previously described [23,24].

To prepare the cRNAs of *HERG*, *KCNQ1* and *mlsK*, plasmids containing these channel genes were first linearized by restriction endonucleases. The pSP64 plasmids containing the *HERG* gene

were linearized with *EcoRI*. The pEXO plasmids containing the *mKCNQ1* clone were linearized with *BamHI*. The original pBlueScript SK⁻ vector containing the *mIsK* gene was firstly sub-cloned into the pGEM-HE vector. Then the pGEM-HE vector was linearized with *PstI*. Using the linearized plasmids as templates, cRNAs were synthesized *in vitro* by the large-scale T7 mMACHINE mMACHINE transcription kit.

Electrophysiology experiments

Whole-cell currents from oocytes were recorded using the two-microelectrode voltage-clamp technique. Current measurements were performed with a two-microelectrode voltage-clamp system (GeneClamp 500, Axon Instruments, Burlingame, CA, U.S.A.). Voltage and current electrodes (0.4–2 M Ω) were filled with 3 M KCl. Current records were sampled at 0.5 ms intervals after low-pass filtering at 1 kHz. Linear components of capacity and leak currents were not subtracted. All experiments were performed at room temperature (19–23 °C). Fitted K_d values were obtained after calculating the fraction current left over after application of several toxin concentrations in different oocyte experiments.

Solutions

The ND-96 solution (pH 7.5) contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM Hepes, supplemented with 50 mg/l gentamycin sulphate (Schering-Plough, Brussels, Belgium) when incubated. The calcium-free ND-96 solution was identical with the ND-96 solution without CaCl₂. The high potassium solution contained 96 mM KCl, 2 mM NaCl, 1 mM MgCl₂, 1.8 mM CaCl₂ and 5 mM Hepes neutralized to pH 7.5 with KOH.

Homology modelling

The amino acid sequence of BmTx3 was sent to the 3D-PSSM server (<http://www.sbg.bio.ic.ac.uk/~3dpssm/>) to search for the best template. The scorpion toxin TyK α was found to share the highest identity (57%) with BmTx3 [18]. The sequences of BmTx3 and TyK α were aligned by Multiple Sequence Alignment Software (ClustalX) [25], and the alignment result was directly submitted to the SWISS-MODEL server for modelling [26]. Energy minimization (Gromos96) and simulated annealing cycles were run. Taking into account the deviation of the model from the template structure and the distance trap value used for framework building, the SWISS-MODEL program computed a confidence factor for each atom in the model of the structure.

Phylogenetic tree

The amino acid sequences of different scorpion toxins were aligned by Multiple Sequence Alignment Software (ClustalX) [25]. Based on this sequence alignment, a rooted Unweighted Pair Group Mean Average (UPGMA) phylogenetic tree was constructed with Molecular Evolutionary Genetics Analysis Software Version 2 (MEGA2) [27]. Evolutionary distances among different toxins were computed by the Poisson Correction Distance method.

RESULTS

Purification and synthesis of BmTx3

As detailed in the Experimental section, a HERG-channel blocker was purified from scorpion *BmK* venom by gel-filtration chromatography, anion-exchange chromatography, RP-HPLC and cation-exchange HPLC (Figures 1A and 1B). The first step of the

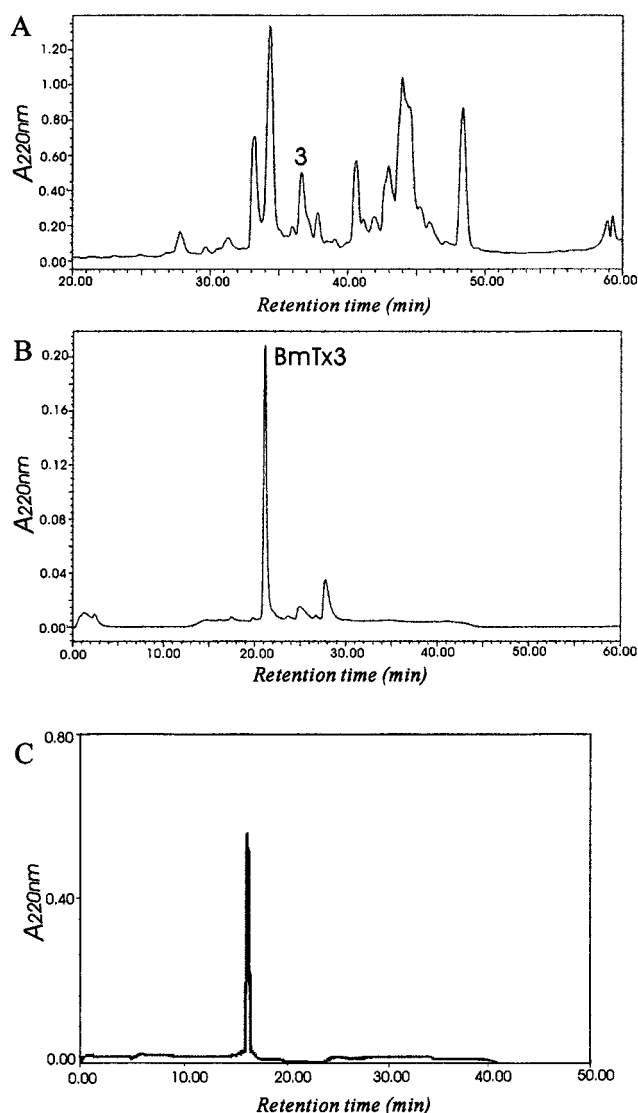


Figure 1 Purification of BmTx3 and chromatographic comparison of native and synthetic BmTx3

(A) RP-HPLC profile. The breakthrough fraction from the DEAE–Sephacel column was applied to a semi-preparative C₁₈ column equilibrated with buffer A (0.1% TFA in water). The column was eluted with a linear gradient by two steps: 0–36% buffer B (70% acetonitrile in buffer A) for 36 min and 36–46% buffer B for 15 min. (B) Cation-exchange chromatographic profile of Mono-S column. Fraction 3 from the C₁₈ column was applied to the Mono-S column equilibrated with ethanoic acid/sodium ethanoate buffer (pH 4.3). Proteins were eluted with a linear NaCl gradient (0–1 M) in the equilibration buffer. The component of the first peak was BmTx3. (C) Native and synthetic BmTx3 were mixed and consecutively loaded onto a C₁₈ HPLC column. Only one peak could be observed, indicating that these peptides were identical.

Edman degradation of this toxin could not be processed because the N-terminus was blocked by the residue pyroglutamate. Similar cases can be found in some other scorpion toxins [28,29]. After the pyroglutamate was removed by pyroglutamate aminopeptidase, the whole toxin sequence was successfully determined by the Edman degradation. The obtained sequence was found to be completely identical with that of a previously characterized toxin BmTx3, an A-type K⁺-current blocker [7].

In order to obtain more material for further functional analysis, BmTx3 was synthesized as previously described [7]. The native and synthetic BmTx3 were co-eluted on RP-HPLC, indicating their identity (Figure 1C).

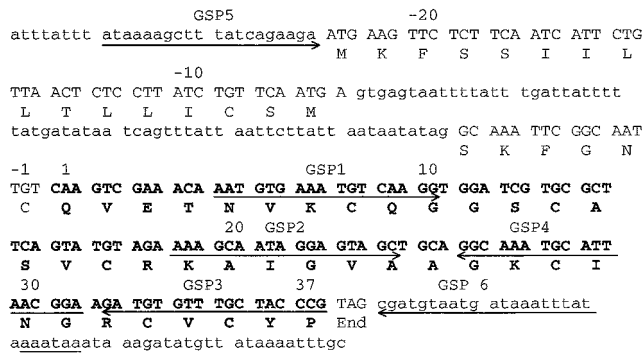


Figure 2 Gene structure of BmTx3

The two exons are denoted with capital letters, whereas the UTR and the intron are denoted with lower-case letters. The polyadenylation signal is underlined. The deduced amino acid sequence is annotated below the nucleotide sequence and numbered according to the mature toxin characterized previously. The deduced mature peptide is written in bold. Primers for RACE and genomic gene amplification are also indicated above the sequence (GenBank® accession numbers AF541980 and AY156725 for cDNA and genomic gene of BmTx3 respectively).

cDNA and genomic gene cloning

According to the determined protein sequence of BmTx3, gene-specific primers were designed to amplify the cDNA of BmTx3 and the corresponding genomic gene. The complete cDNA sequence of BmTx3 was obtained by overlapping the 3' and 5' partial cDNA sequences (Figure 2). The open reading frame encoded a signal peptide of 22 residues and a mature peptide of 37 residues. The amino acid sequence of the signal peptide was almost similar to the sequences depicted for the Aa1 analogue precursors [11]. The deduced amino acid sequence of BmTx3 was consistent with the chemically determined sequence, except for the fact that the N-terminal residue pyroglutamate, instead of glutamine, appeared in the determined sequence. This was also observed for the Aa1 analogues. Obviously, glutamine was post-translationally modified to pyroglutamate via a condensation of the α -amino group of the glutamine residue with the γ -carboxy group of this glutamine residue. This kind of modification was also found in ChTx, BmTx1, BmTx2 and other α -KTxs [22,30]. The polyadenylation signal, AATAA, was found in the 3'-UTR at position 24 upstream from the polyadenylated sequence.

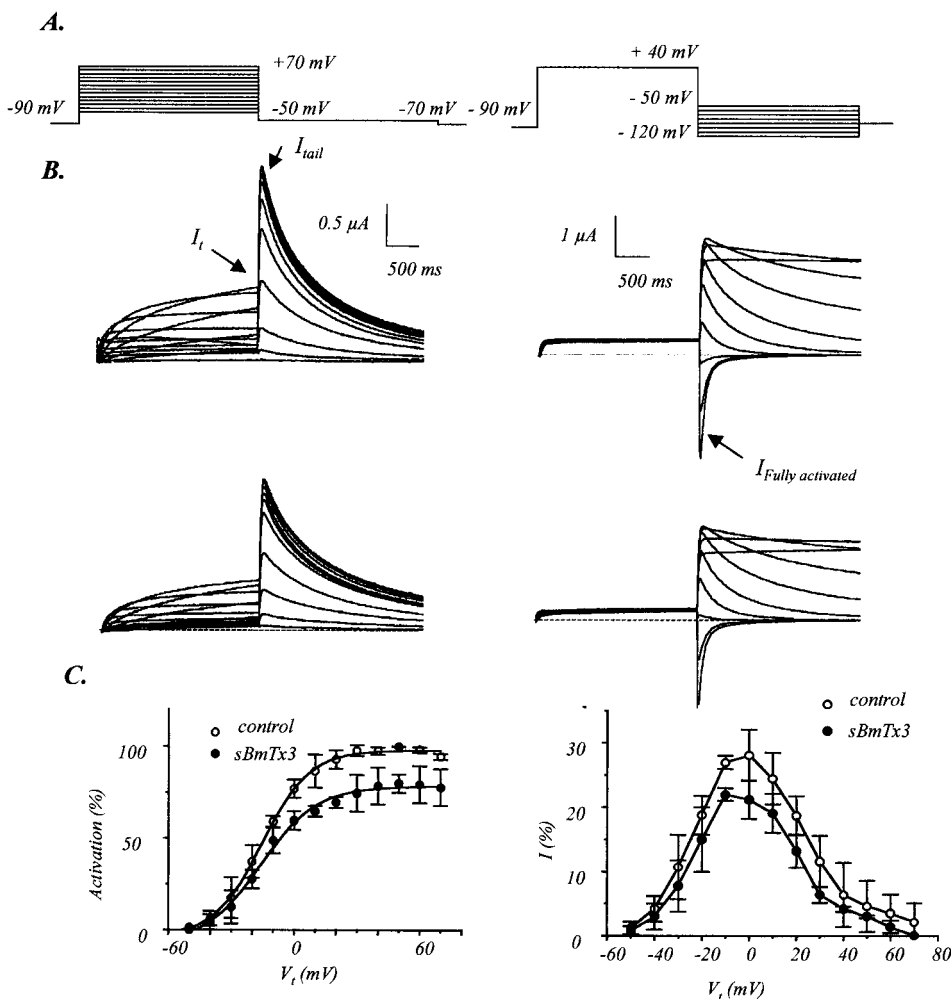


Figure 3 HERG currents recorded in *Xenopus* oocytes

(A), (B) Left-hand side: HERG currents (I_t) were evoked by application of 2 s test pulses ranging from -50 to +70 mV in 10 mV increments from a holding potential of -90 mV. Peak I_{tail} values were measured at a potential of -70 mV. Right-hand side: the oocyte membrane was depolarized from a holding potential of -90 mV to +40 mV to activate the channels fully. Thereafter, repolarization steps ranging from -50 to -120 mV in 10 mV increments were applied. sBmTx3 inhibition (500 nM) of HERG channels (lower panels), with protocols as detailed in A. (C) Left-hand side: activation curves of HERG channels in control (○) and in the presence of 500 nM sBmTx3 (●). The curves were constructed using the I_{tail} after test pulses to various V_t and the I_{tail} values were normalized by the maximum I_{tail} after a test pulse of 40 mV ($n=3$). Right-hand side: Current-voltage (I - V_t) relationship where I_t was measured at the end of each test pulse in B. The I_t were normalized using the maximum $I_{fully activated}$ recorded at -50 mV in the same oocyte ($n=3$).

The genomic DNA of BmTx3 is composed of two exons interrupted by an intron of 65 bp (Figure 2). The intron, with a consensus splicing site of GT at the 5' end and AG at the 3' end, was inserted into the signal-peptide-encoding region at position -6 upstream from the mature toxin. The gene structure of BmTx3 was similar to those of other scorpion toxins previously reported and highly related to those of Aa1 analogues [11,21,31–33]. Most precursors of the scorpion toxins acting as K⁺-channel blockers have a putative signal peptide of 20–28 residues. Their genes are normally composed of two exons interrupted by an intron of 60–130 bp inserted into the signal peptide encoding region near the C-terminus. The introns were in accordance with the GT/AG splicing junctions and rich in A + T content (> 80%), in contrast with the exons (< 60%) [34].

HERG-channel-block activity

Figure 3 illustrates the expression of HERG channels in *Xenopus laevis* oocytes with their typical characteristics described by Sanguinetti et al. [35]. The voltage-clamp protocols are shown at the top of Figure 3(A). The synthetic BmTx3 (sBmTx3, 500 nM) reversibly blocked the small outward currents (I_t), the large outward tail currents (I_{tail}), and the inward currents ($I_{fully\ activated}$) as illustrated in Figure 3(B). To measure the voltage-dependence of the channel activation (Figure 3C, left), the activation curve was constructed using the peak amplitudes of the I_{tail} values as shown in Figure 3(B). The I_{tail} amplitudes (by each test potential V_t) were normalized by the maximum I_{tail} recorded at 40 mV to estimate the percentage of ion channels that were activated. This activation curve could be well fitted using a simple Boltzmann function (percentage activated = $100/\{1 + \exp[(V_{1/2} - V_t)/S]\}$, where $V_{1/2}$ represents the half-maximal potential and S is the slope factor). The inhibition effect of 500 nM sBmTx3 showed no shift in the $V_{1/2}$ of the channel, with values of -15.3 ± 0.8 mV and -15.9 ± 1.8 mV and slope factors of 11.4 ± 0.6 mV and 13.4 ± 1.5 mV for the control situation and in the presence of toxin respectively ($n = 3$). The current–voltage (I_t – V_t) relationship of the test pulses is bell-shaped both in control and in the present of sBmTx3 (Figure 3C, right). I_t was measured at the end of each test pulse in Figure 3(B) (left part). I_t values were normalized using the maximum $I_{fully\ activated}$ recorded at -50 mV in the same oocyte ($n = 3$). These results indicate that both the activation and inactivation processes increase with depolarization and are unaffected by the toxin.

The concentration–response relationship of sBmTx3 on the HERG currents has been analysed (Figure 4). Oocytes have been repeatedly pulsed to 0 mV and peak I_{tail} amplitudes have been evoked at -70 mV. Increasing concentrations of sBmTx3 were applied to oocytes. The percentage of the remaining tail current at the steady-state of the toxin blockade has been plotted against the toxin concentration on a logarithmic scale, and the data have been fitted using the function $\{1 + (K_d/[toxin])^h\}^{-1}$. The K_d value was 1.9 ± 0.14 μ M and the Hill coefficient was 1.3 (each data point represents three experiments on different oocytes).

We also analysed the effect of a specific deletion mutant of BmTx3, named BmTx3-YPdel, which is devoid of Tyr³⁶ and Pro³⁷ at the C-terminus. In contrast with wild-type BmTx3, BmTx3-YPdel did not block A-currents at concentrations up to 35 μ M, indicating that this deletion results in the loss of its A-type K⁺-current block activity [20]. Interestingly, we found that this mutant had a similar affinity (K_d value of 3.9 ± 0.2 μ M) for HERG channels as compared with the wild-type BmTx3 (K_d value of 1.9 ± 0.1 μ M). For both the wild-type toxin and the mutant, full channel block could not be achieved (Figure 4).

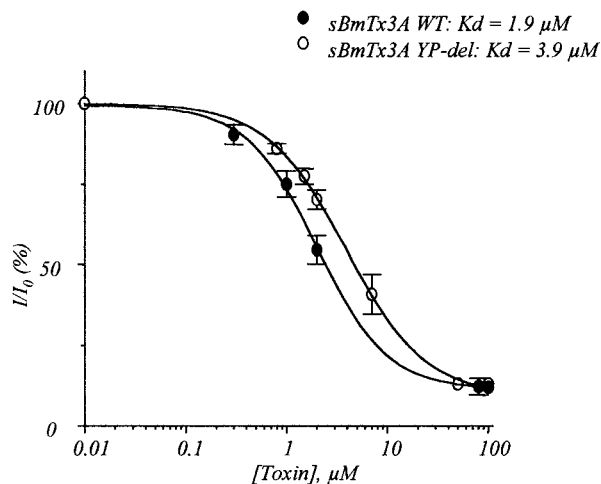


Figure 4 Dose–response relationship of sBmTx3 and sBmTx3-YPdel inhibition of HERG currents

Peak I_{tail} amplitudes in Figure 3(B) evoked at -70 mV were used to construct the concentration–response curve. HERG channels are blocked by sBmTx3 and sBmTx3-YPdel with a K_d values of 1.9 μ M and 3.9 μ M respectively (Hill = 1.3 and Hill = 1.2 respectively). Each point represents the mean \pm SD from three oocytes.

Selectivity pattern of BmTx3

Because the HERG channel is inherent with the characteristics of both the Kv-type and inward rectifier channels, we investigated further the activity of sBmTx3 on other Kv channels (Kv1.1, Kv1.2, Kv1.3, KCNQ1 and KCNQ1 + mIsK) and an inward rectifier channel (Kir2.1). No effects on any of these channels could be observed even at a concentration up to 5 μ M sBmTx3 (Figure 5), at which concentration, most HERG currents could be inhibited. Thus the selectivity pattern of BmTx3 seems to be represented by the HERG and A-type K⁺ currents.

DISCUSSION

Based on the primary sequence structures, scorpion toxins active on K⁺ channels are classified into three families, namely α -KTx, β -KTx and γ -KTx [29]. BmTx3 was grouped into subfamily 15 of α -KTx, together with AmmTx3, Aa1 and Aa1 analogues [7]. Aa1 and AmmTx3 were also found to be able to block A-type K⁺ currents [9,10]. To date, a total of 27 HERG blockers have been identified from scorpion venoms, and they are all grouped into γ -KTxs [36]. BmTx3 is the first reported α -KTx found to also have γ -KTx activity. In the view of the evolution of toxins, we propose that BmTx3 might represent an evolutionary transitional member between α -KTx and γ -KTx families. A rooted phylogenetic tree was therefore constructed to study the evolutionary distances among classical Kv and/or BK_{Ca} blockers (α -KTx1, 2, 3), α -KTx15 and classical HERG blockers (γ -KTx) (Figure 6). BmTx3 takes an intermediate position between classical Kv and/or BK_{Ca} blockers and classical HERG blockers in the tree. Hence this result could indeed support our proposal.

Given the fact that the functional face of classical α -KTx is located on the β -sheet face, whereas the functional face of γ -KTx shifts to the α -helix face, we wondered whether BmTx3 contained both of these two functional faces. Thus it was useful to construct a model of BmTx3 based on the NMR structure of a homologous toxin, TyK α [18]. This model represents the conserved cysteine-stabilized α/β scaffold (CS α/β) present in most known scorpion toxins [3]. We compared this structure model with the structures

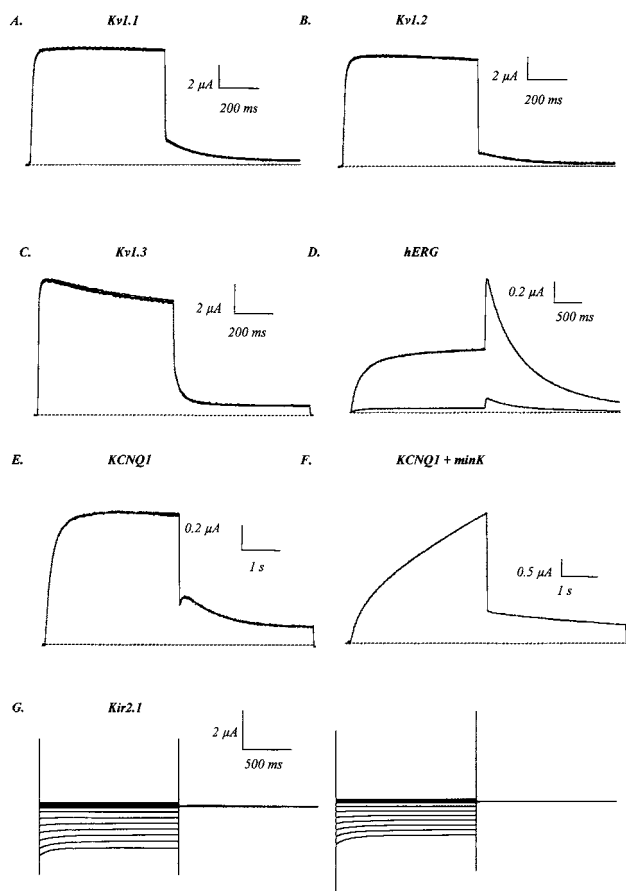


Figure 5 Selectivity of sBmTx3 towards different K⁺ channels

Effect of 5 μM sBmTx3 on Kv1.1 (A), Kv1.2 (B), Kv1.3 (C), hERG (D), KCNQ1 (E), KCNQ1 + mlsK (F) and Kir2.1 (G) channels expressed in *Xenopus* oocytes. Kv1-type currents were evoked by depolarizing the cell to 0 mV from a holding potential of -90 mV for 2 s. Tail currents were recorded at -50 mV. hERG currents were induced by depolarizing the oocyte membrane to 0 mV from a holding potential of -90 mV for 2 s and then clamped back to -70 mV. For KCNQ1 channels (alone), oocytes were clamped from a holding potential of -90 mV to 0 mV for 4 s and clamped back to -50 mV. KCNQ1 + mlsK channels were clamped from a holding potential of -90 mV to 20 mV and clamped back to -50 mV. For Kir2.1 channels, current recordings were performed in high-potassium solution. Traces in the absence (left) or presence (right) of sBmTx3 were elicited (from a holding potential of 0 mV) by steps from 60 mV to -160 mV in 20 mV increments. Cells were clamped back to 0 mV.

of ChTx, the most well-known toxin of the α-KTx family and BeKm-1, another well-known member of the γ-KTx family (Figure 7). Like ChTx, BmTx3 has a putative functional dyad composed of Lys²⁷ and Tyr³⁶ residues at its β-sheet face. In ChTx and many other α-KTx toxins, the functional dyad composed of an aromatic residue (Tyr/Phe) and a lysine residue (always Lys²⁷) plays a central role in toxin-channel interaction (Figure 7B) [6]. This should be the same in the case of BmTx3. For the hERG channel blocker BeKm-1, two functionally crucial residues (Lys¹⁸ and Arg²⁰) have been determined at the opposite α-helix face. The corresponding residues (Arg¹⁸ and Lys¹⁹) could be found in BmTx3 (Figure 7C). Therefore we propose that BmTx3 might have two separate functional faces for different channel-blocking functions, namely Face A (composed of Arg¹⁸ and Lys¹⁹ at the α-helix side) corresponding to the hERG-blocking activity, and Face B (containing the dyad at the β-sheet side) corresponding to the A-type K⁺-current-blocking activity (Figure 7A).

As has been shown for many *Shaker*-type K⁺ toxins, the disruption of the functional dyad was found to eliminate their K⁺-

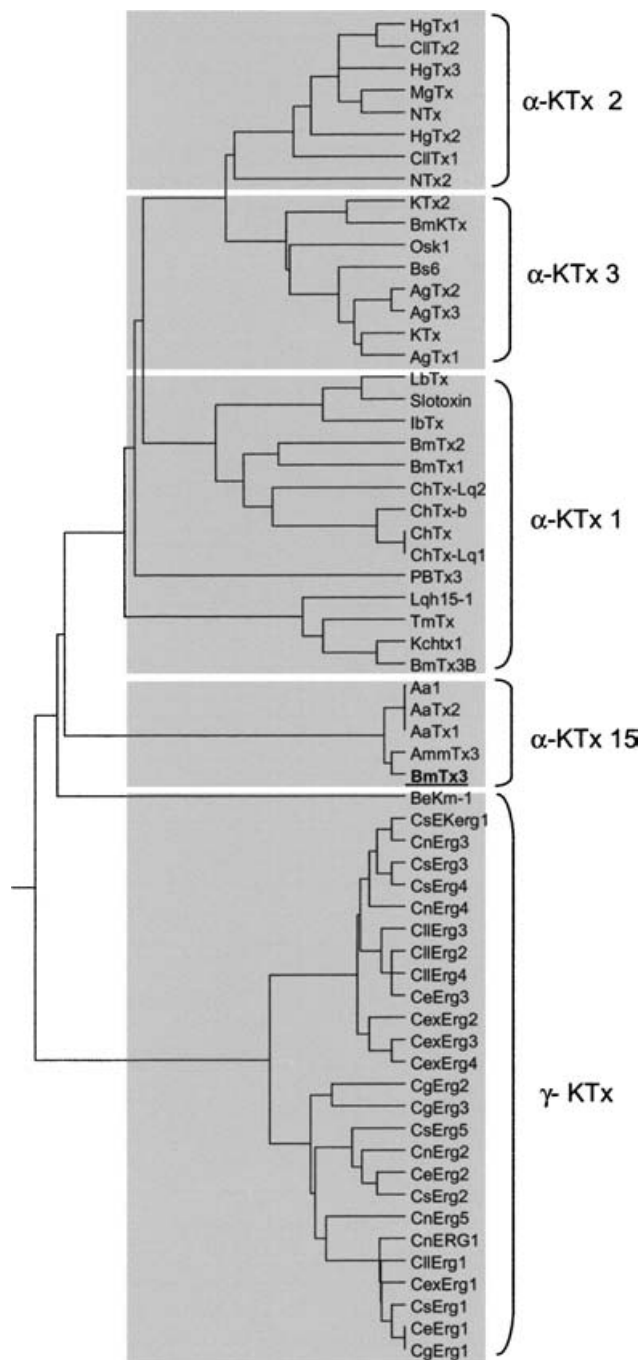


Figure 6 Phylogenetic tree

A rooted Unweighted Pair Group Mean Average (UPGMA) tree was constructed to study the evolutionary distances among classical Kv and/or BK_{Ca} blockers (α-KTx1, 2, 3), α-KTx15 and classical hERG blockers (γ-KTx). BmTx3 (bold and underlined) took a transitional position between classical Kv and/or BK_{Ca} blockers and classical hERG blockers in the tree.

channel-blocking activity [6,37]. A similar effect was observed in the case of BmTx3. It has already been reported that the deletion of Tyr³⁶, part of the dyad, and Pro³⁷ in BmTx3 results in the loss of the A-type K⁺-current-blocking activity [20]. According to our two functional faces hypothesis, the hERG-blocking activity of this BmTx3-YPdel should remain, as its Face A was still intact. This prediction was verified by the fact that the affinity of the deletion mutant for the hERG channels decreased only 2-fold compared with the wild-type toxin (Figure 4).

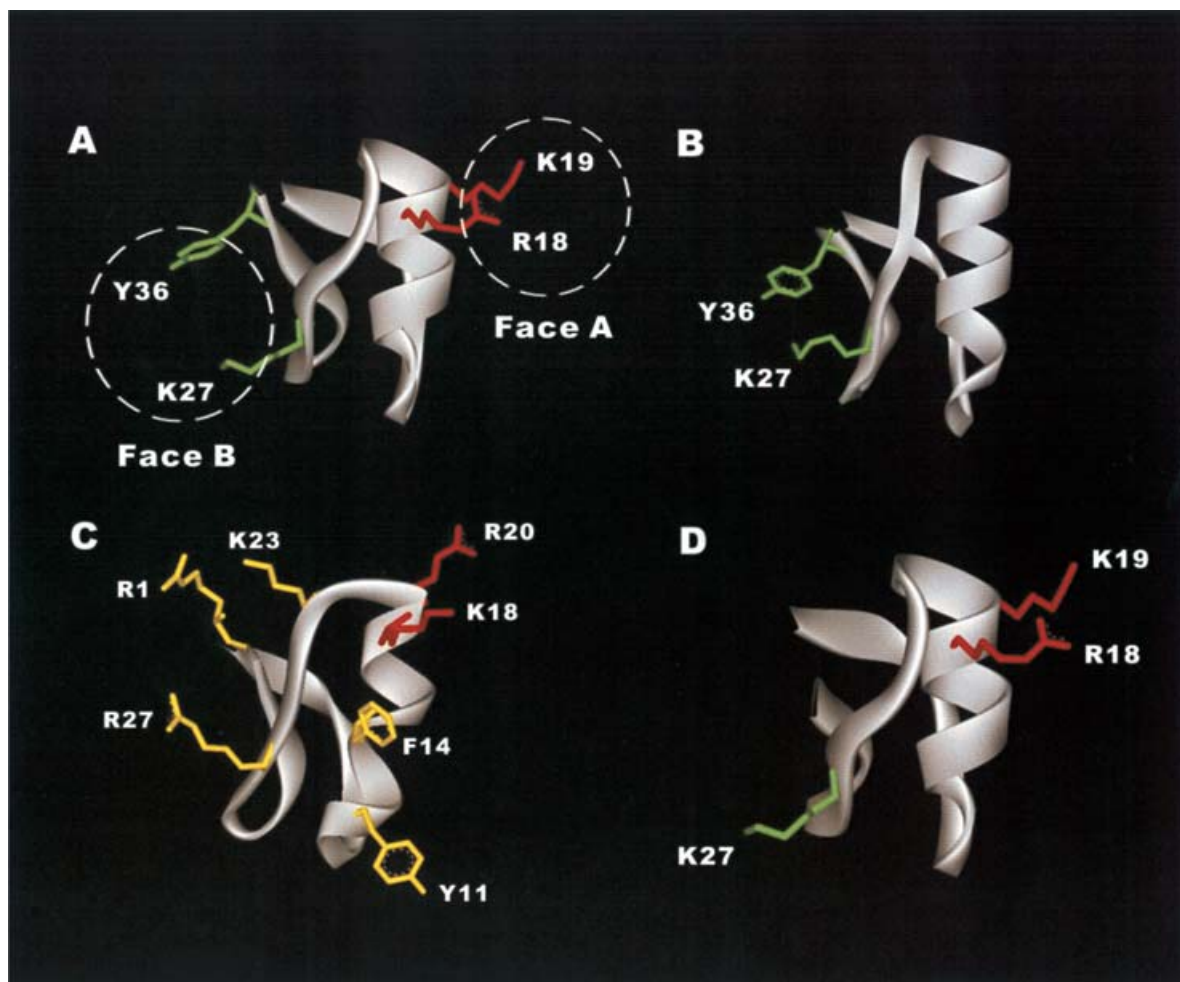


Figure 7 View of the two functional faces of BmTx3

(A) BmTx3 with two putative functional faces: Face A composed of Arg¹⁸ and Lys¹⁹ in red is located at the α -helix side; Face B containing a functional dyad (Lys²⁷–Tyr³⁶ in green) is located at the β -sheet side. (B) ChTx with the functional dyad (Lys²⁷–Tyr³⁶ in green). (C) BeKm-1 with the most important residues (in red) and moderately important residues (in yellow) for the HERG-blocking activity. (D) BmTx3-YPdel with the intact Face A, but broken Face B.

The inter-atomic distance between the C α -atom of Lys²⁷ and the benzene ring of Tyr³⁶ in BmTx3 (8.5 Å, where 1 Å = 0.1 nm) was larger than the value (6.6 ± 1.0 Å) in *Shaker*-type toxins [6]. This long distance could not meet with the criteria of *Shaker*-type-blocking activity, but might still be suitable for the A-type K⁺-blocking activity. Given the fact that, except for residues Lys¹⁸ and Arg²⁰, BeKm-1 has other moderate important residues which are absent in BmTx3 (shown in yellow in Figure 7C), we can understand the relatively low affinity of BmTx3 for HERG channels (K_d value of 1.9 μ M) compared with that of BeKm-1 (K_d value of 6.3 nM) [16].

Recently, three scorpion toxin–channel interaction modes were thoughtfully discussed [3,15]. The authors concluded that the toxins from subfamilies α -KTx1–4 and α -KTx6 mainly used the β -sheet face (Face B) to interact with the pore region of Kv1.x, K_{Ca}1.1 and K_{Ca}3.1 channels, while toxins from the γ -KTx family used the α -helix face (Face A) to interact with the turret region of the HERG channel. However, no scorpion toxin was reported to have two functional faces simultaneously. To our knowledge, the closest comparison that can be made with respect to two functional faces in one toxin molecule is the ‘Janus Ligand Hypothesis’, which postulates that there are two distinct recognition faces

in some *Conus* peptides for the nAChR (nicotinic acetylcholine receptor) [38]. However, these ‘Janus’ ligands target the α and β subunit of the nAChR respectively, whereas BmTx3 is capable of docking on two quite different K⁺ channels.

In conclusion, we believe that our work sheds light on a new structure–function relationship of scorpion toxins. The implication is that BmTx3 can be considered as a transitional member in the evolution between α -KTx and γ -KTx families of scorpion toxins.

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