Molecular basis of the mammalian potency of the scorpion α-like toxin, BmK M1

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ABSTRACT

In-depth structure-function studies of voltage-gated Na⁺ channels and peptide toxins are continuously increasing our understanding of their interaction. In this study, an effective yeast expression system was used to study the role of 14 N- and C-terminal residues from the α-like toxin BmK M1 from the Chinese scorpion Buthus martensii Karsch. With the use of site-directed mutagenesis, all of these residues were individually substituted by one or more amino acids, resulting in a total of 19 mutants. These were then subjected to a bioassay on mice, an elaborate electrophysiological characterization on three cloned voltage-gated Na⁺ channels (Nav1.2, Nav1.5, and para), and a circular dichroism analysis. Our results reveal large mutant-dependent differences that emphasize important and specific roles for the studied residues. By mutating single amino acids, we were able to redirect the α-like characteristics of BmK M1 (active on both mammals and insects) to either much higher mammal specificity or, in a few cases, total insect specificity. This study therefore represents a thorough mapping and elucidation of three epitopes that underlie the molecular basis of the mammalian and insecticidal potency of the scorpion α-like toxin, BmK M1 on voltage-gated Na⁺ channels.

Key words: voltage-gated Na⁺ channels

Voltage-gated Na⁺ channels (VGSCs) are transmembrane protein complexes that are composed of a pore-forming α-subunit (260 kDa) associated with up to four auxiliary β-subunits (1–3). Until this date, nine mammalian (Na₁.1-Na₁.9) and three invertebrate VGSCs (fruit fly, housefly, and cockroach) have been cloned (4, 5). Despite their similarity in electrophysiology and ion conductance, primary structure, and allocation of all functional domains, the insect and vertebrate VGSCs are pharmacologically distinguishable (6).
Since VGSCs play an essential role in the signal transduction process of membranes of most electrically excitable cells, in particular in the rising phase of an action potential, they are a welcome target for toxins of various organisms and different chemical structures. Toxins have been used to describe seven different receptor sites on the α-subunit, all of which are closely linked to specific effects on the function of VGSCs (7). The scorpion α-toxins, funnel web spider toxins, and the sea anemone α-toxins all bind to site 3, possibly by interfering with the outward movement of the voltage-sensing segment of the VGSC, which results in delayed inactivation of the channel (8–11). For our study, this site is the most important one.

According to their different pharmacological preferences, the scorpion α-toxins can be divided into three subgroups: classical α, α-like, and insect α-toxins (12, 13). Classical α-toxins are highly toxic to mammals (e.g., AaH II from Androctonus australis Hector, Lqh II from Leiurus quinquestriatus hebraeus) and insect α-toxins are especially toxic for insects (e.g., Lqh α IT from Leiurus quinquestriatus hebraeus) (14–16). The α-like toxins act on both mammals and insects but do not bind to rat synaptosomes despite a high toxicity by intravenous injection (e.g., BmK M1, Lqh III from Leiurus quinquestriatus hebraeus) (17–19).

BmK M1 is a scorpion α-like toxin from the eastern Asian scorpion Buthus martensii Karsch (Fig. 1). This toxin is composed of 64 amino acids cross-linked by four disulphide bridges and has been the subject of several thorough studies (17, 19–24).

By using this particular α-like toxin as a starting point, we tried to find the epitopes that determine the target specificity of this toxin for different types of VGSCs. A recent study has described the molecular basis of the high insecticidal potency of scorpion insect α-toxins by transferring two distinct domains of the insect specific α-toxin Lqh α IT to the mammal specific α-toxin AaH II (25). These two distinct domains consist of a conserved “core domain” formed by amino acids of the loops connecting the secondary structure elements of the molecule core and a variable “NC domain” formed by the five-residue-turn (residues 8-12) and the C-terminal segment (residues 56-64). Together with previous studies that had already revealed important information on the putative functional surfaces of Lqh α IT (importance of Tyr10, Phe17, Lys6, Arg18, Lys62, Arg64, and the structural reconfiguration of the C tail) and BmK M1 (the conserved hydrophobic surface, residues 8-12 interaction with the C tail), a general idea is being formed about the interaction mechanisms of these toxins with their mammalian and insect targets (23, 24, 26–29).

Our study confirms some of the previously obtained results but also contradicts a recently proposed binding mechanism responsible for VGSC subtype selectivity of scorpion toxins (25). We also provide a refinement of a previous study where two epitopes were reported to be important (25). Our results clearly indicate that an extra epitope, situated at the N terminus, also contributes to the insect or mammal selectivity of BmK M1. As a consequence, a new definition of the pharmacologically important epitopes is provided. To this end, 14 novel C- and N-terminal residues were mutated to one or more other amino acids and by doing so, we succeeded in redirecting the α-like characteristics of BmK M1 (active on both mammals and insects) to either much higher mammal specificity or, in a few cases, total insect specificity. For the first time, this study combines 1) the effective expression of a large number of toxin mutants with a bioassay on mice; 2) a detailed electrophysiological analysis on three cloned VGSCs, Na,1.2 (rat), Na,1.5 (human) and para (fruit fly); 3) a CD analysis; and 4) the use of two important pharmacological
parameters, potency, and maximum efficacy, necessary for explaining the mutagenesis data: potency equals the affinity of the toxin for the VGSC, whereas maximum efficacy represents the maximum $\alpha$-effect at a given concentration.

MATERIALS AND METHODS

Strains, materials, and animals

The *Escherichia coli* strain TG1 was used for the production of plasmids. The yeast strain *Saccharomyces cerevisiae* S-78 (Leu2, Ura3, Rep4) and expression vector pVT102U/R were used for expression of both the wild-type and mutated BmK M1. Restriction endonucleases and T4 DNA ligase were obtained from Roche Applied Science (Mannheim Germany). Primers were synthesized by Sangon (Shanghai, China). Taq DNA polymerase and Klenow fragment were obtained from MBI. CM32-cellulose cation-exchange and Sephasil® peptide C18 reversed-phase (12-µm ST4.6/250) columns were from Whatman and Amersham Biosciences AB (Uppsala, Sweden), respectively. All other chemicals were at least analytical grade and were purchased from Merck or Sigma. The mice used for the bioassay were ICR mice from the Beijing Center for Experimental Animals.

Site-directed mutagenesis of BmK M1

The locations of the residues selected for mutagenesis are indicated in Fig. 1. The cDNA of BmK M1 was previously cloned and inserted into pVT102U/$\alpha$-BmK M1, two primers were designed: primer 1 (5'-CGTCTAGATAAAAGAAAAGGTTCTCGGGATGCTTATATTGCC-3'), including a KEX2 protease linker and an *Xba*I restriction site and primer 2 (5'-CGAAGCTTTTAATGGCATTTTCCTGGTAC-3') with a *Hind*III site.

The mutagenic primers used to generate the desired mutations were as follows:

DeletionV1: 5'-CGTCTAGATAAAAGAAAATCTCGGGATGCTTATATTGCC-3';
R2A: 5'-CGTCTAGATAAAAGAAAATCTGTTGAAGATGCTTATATTGCC-3';
R2E: 5'-CGTCTAGATAAAAGAAAATCTGTTGCTGCTTATATTGCC-3';
D3A: 5'-CGTCTAGATAAAAGAAAATCTGTTGGCGTGCTTATATTGCC-3';
D3R: 5'-CGTCTAGATAAAAGAAAATCTGTTGGCCGATGCTTATATTGCC-3';
D3N: 5'-CGTCTAGATAAAAGAAAATCTGTTGGCCGATGCTTATATTGCC-3';
I6G: 5'-CGTCTAGATAAAAGAAAATCTGTTGGAGCTGCTTATGCTTATATTGCC-3';
P9N: 5'-CGTCTAGATAAAAGAAAATCTGTTGGAGCTGCTTATATTGCC-3';
P9N/H10Y: 5'-CGTCTAGATAAAAAGAAATTCTGTTCGGATGCTTATATTGCCAAGAA CTACAACTGTGTAT-3';

H10Y: 5'-
CGTCTAGATAAAAAGAAATTCTGTTCGGGATGCTTATATTGCCAAGCCTACAA CTGTGTAT-3';

G34A (+): 5'-GCTAAGAGTGCTTATTGCCAA-3';

G34A (-): 5'-TTG GCAATAAGCACTCTTAGC-3';

G43A (+): 5'-GGTAAATACGCTAATGGCTGC-3'; G43A (-):
5'-GCAGCCATTAGCGTATTTACC-3';

L51G (+): 5'-TGCATAGAGGGT CCCGATAAT-3';

L51G (-): 5'-ATTATCGGGAC CCTCTATGCA-3';

P52S (+): 5'-ATAGAGTTGAGTGATAATGTA-3';

P52S (-): 5'-TACATTATC.ACTCAACTCTAT-3';

I57G: 5'-CGAAAGCTTTTAATGGGCATTTTTCCTGGTACTCGACCGGTACATT ATC-3';

V59G: 5'-CGAAGCTTTTAATGGGCATTTTTCCTGGACCTCGAATCGGTAC-3';

P60A: 5'-CGAAGCTTTTAATGGGCATTTTTCCTAGCTACTCGAATCGG-3';

P60G: 5'-CGAAGCTTTTAATGGGCATTTTTCACCTACTCGAATCGG-3';

G61A: 5'-CGAAG CTTTTAATGGGCATTTTAGCTGGTACTCGAATCGG-3'.

With the use of pVT102U/α-BmK M1 (rBmK M1) as template along with primers 1,2 and the mutagenic primers, mutants deletionV1, R2A, R2E, D3A, D3N, D3R, I6G, P9N, P9N-H10Y, H10Y, I57G, V59G, P60A, P60G, and G61A were created by one-step PCR. Mutants G34A, G43A, L51G, and P52S were obtained by three-step PCR. All PCR products were purified by gel excision.

Expression and purification of mutants

After digestion with XbaI and HindIII, the mutated cDNA gene was inserted into the plasmid pVT102U/α and transformed into E. coli TG1 competent cells. The recombinant plasmid pVT102U/α mutant was extracted, sequenced, and transformed into S. cerevisiae S-78 cells using the LiCl method (30). The expression of the mutants was carried out using a previously described procedure (22). After fermentation, the supernatant of the culture was adjusted to pH 4.2 with acetic acid. The sample was directly applied onto a CM32-cellulose cation-exchange
column (2.8×14 cm), which was equilibrated with 0.1 M sodium acetate at a flow rate of 1 ml/min. Upon reaching a steady baseline, the column was washed by stepwise elution with 0.2, 0.3, and 0.5 N NaCl equilibration buffer. Guided by electrophoresis, either the 0.3 N or 0.5 N NaCl fractions were directly applied onto a Sephasil peptide C\textsubscript{18} reversed-phase column. The sample volume used for reversed-phase chromatography was 50 ml each time, and the volume of the C\textsubscript{18} column was 4 ml. Buffer A contained 0.1% trifluoroacetic acid in water; buffer B contained 0.1% trifluoroacetic acid in acetonitrile. The C\textsubscript{18} column was eluted with a linear gradient of 0-80% buffer B for 15 column volumes. Reversed-phase chromatography was carried out using an ÄKTA purifier chromatography system (Amersham Biosciences AB).

**Molecular mass determination**

The molecular masses of the purified mutants were obtained using a triple quadrupole LC/MS/MS mass spectrometer (Perkin-Elmer Sciex Instruments) equipped with an electro spray ionization source. Calculations were performed using the program provided by the manufacturer. Masses were double-checked on a Thermofinnigan Deca XP ion-trap LC/MS.

**Bioassay**

With the use of 0.9% NaCl as a negative control and rBmK M1 as a positive control, the toxicity of the mutants was determined in mice (male, specified pathogen free level, 18–20 g of body wt.). Various doses of toxin mutants were dissolved in 0.9% NaCl and injected into the mice through the tail vein. Survival times (times between injection and death), reaction, and doses were recorded. Evaluation of toxicity was based on the determination of LD\textsubscript{50} (the dose capable of statistically killing 50% of the mice) according to the method of Meier and Theakston (31). The body weight, injection dose, and survival time were recorded for calculation of the LD\textsubscript{50}.

**CD measurements**

Samples used for analyses were dissolved in 25 mM Tris-HCl (pH 8.0) buffer at a concentration of 1.0 mg/ml. CD spectra were recorded on a Jasco J-720 spectropolarimeter. Spectra were run at 25°C from 250 to 200 nm using a quartz cell of 0.5 mm in length. Data were collected at 1.0 nm intervals with a scan rate of 50 nm/min. All CD spectra resulted from averaging four scans. The final spectrum was corrected by subtracting the corresponding baseline spectrum obtained under identical conditions. Spectra were smoothed by the software of the instrument. The secondary structure content was estimated by standard Jasco CD analysis.

**Expression in Xenopus laevis oocytes, electrophysiological recordings, and analysis**

For the expression in *Xenopus* oocytes, the hH1 (Na\textsubscript{v}1.5) and β\textsubscript{1} genes were subcloned into pSP64T (32). For in vitro transcription, hH1/pSP64T was first linearized with *Xba*I and β\textsubscript{1}/pSP64T with *EcoRI*. Next, capped cRNAs were synthesized from the linearized plasmid using the large-scale SP6 mMESSAGE-mMACHINE transcription kit (Ambion). The para/pGH19-13-5 vector, tipE/pGH19 vector, and rBrainII (Na\textsubscript{v}1.2)/pLCT1 vector were linearized with *Not*I and transcribed with the T7 mMESSAGE-mMACHINE kit (Ambion) (5, 33–34).

The harvesting of oocytes from anesthetized female *Xenopus laevis* frogs was as described previously (35). Oocytes were injected with 50 nl of cRNA at a concentration of 1 ng/ml using a
Drummond micro-injector. The solution used for incubating the oocytes contained (in mM) NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 2, and HEPES 5 (pH 7.4), supplemented with 50 mg/l gentamycin sulfate and 180 mg/l theophyllin.

Two-electrode voltage-clamp recordings were performed at room temperature (18-22°C) using a GeneClamp 500 amplifier (Axon Instruments) controlled by a pClamp data acquisition system (Axon Instruments). Whole-cell currents from oocytes were recorded 2-4 days after injection. Voltage and currents electrodes were filled with 3 M KCl. Resistances of both electrodes were kept as low as possible (<0.5 MΩ). Bath solution composition was (in mM) NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 2, and HEPES 5 (pH 7.4). With the use of a four-pole low-pass Bessel filter, currents were filtered at 1 kHz and sampled at 10 kHz. Leak and capacitance subtraction were performed using a P/2 protocol. Current traces were evoked in an oocyte expressing the cloned VGSCs by depolarizations between -70 to 40 mV, using 10 mV increments, from a holding potential of -90 mV.

The degree of fast inactivation was assayed by measuring the I_{10 ms}/I_{peak} ratio, which gives an estimate of the probability for the channels not to be inactivated after 10 ms (36). Depending on the sodium channel, a test voltage was chosen so that I_{10 ms}/I_{peak} was close to zero under control conditions. I_{10 ms}/I_{peak} was measured at the same test voltage after addition of the toxin. Toxin-induced removal of fast inactivation was measured by plotting I_{10 ms}/I_{peak} as a function of toxin concentrations. The following equation was used

\[ I_{10 ms}/I_{peak} = a_0 + \frac{a_1}{1+(EC_{50}/[\text{toxin}])^h} \]

where h is the Hill coefficient, [toxin] the toxin concentration, a₀ the value of I_{10 ms}/I_{peak} obtained at a chosen test voltage under control conditions, and the sum of a₀ and a₁ equals the maximum value of I_{10 ms}/I_{peak} at the chosen test voltage indicating the expected maximum effect of the toxin on fast inactivation. Curve manipulations were performed using pClamp8 (Axon Instruments) and Origin software (Microcal).

RESULTS

Mutation, expression, and purification

Single point mutants were produced by one-step or three-step PCR. The target gene was expressed using the pVT102U/α vector. The expression levels of the mutants were ~1-5 mg/l of culture medium except for the P52S mutant that was only present in a trace amount (Table 1). Expressed mutants were purified by a simple and efficient protocol. One liter of culture (10 g/l yeast extract, 20 g/l bacteriological peptone, 20 g/l glucose, pH after sterilization of 6.5) was harvested and initially purified by chromatography on a CM32 cation-exchange column. The next step of purification was carried out on a C_{18} column. The elution peaks corresponding to target mutants were pooled and lyophilized. The Tricine/SDS-polyacrylamide gels and the mass spectra showed a high purity of the final products. As an example, the entire process for the H10Y mutant is shown in Fig. 2.
Molecular mass

The molecular masses of the purified mutants were measured with the triple quadrupole LC/MS/MS mass spectrometer. The results from the mass spectrometry for all mutants are listed in Table 1. The individual peaks revealed that the molecular masses of the mutants corresponded well with their estimated values (Fig. 2; Table 1). In the case of the D3R mutant, the single mutation of aspartic acid to arginine led to the deletion of the N-terminal segment starting from residue D3 due to the following reason. When the vector pVT102U/α was used, there is a DNA sequence of the MFα leader peptide in front of the cDNA of rBmK M1. The C-terminal residues of this MFα leader peptide are K-R-. When the recombinant protein is excreted, the endoprotease of yeast (KEX2) can recognize the sequence K-R- and consequently cuts the MFα leader peptide to produce the mature protein. However, the mutation D3R gave rise to a sequence of R2-R3- (see Fig. 1), which has a certain similarity to K-R-. This can also be recognized and cut by KEX2. In addition, it has been already identified that, compared with the wild-type BmK M1, the recombinant BmK M1 possesses two additional residues at the N terminus, namely N(-1)-S(0), a contribution from the yeast expression system (22). As a result, the D3R mutation actually produced a mutant with a deletion of five N-terminal residues, N(-1)-S(0)-V1-R2-D3. The mass spectra confirmed this result showing a molecular mass of 6847.8 for this mutant (Table 1), which is in accordance with the estimated molecular mass of rBmK M1 without the five N-terminal residues (6847.7). This implies that the mutation D3R actually is a V1-D2-R3 deletion mutant (Deletion1-3) as listed in Table 1.

Conformational analysis

The CD spectra of rBmK M1 and the mutants that differed in the UV range of 250–200 nm are shown in Fig. 3. The mutants that did not have a significantly changed CD spectrum indicating an unaltered secondary structure are not shown.

Bioassay

The mice showed typical symptoms of envenomation after injection with rBmK M1. The LD50, determined by the method of Meier and Theakston (31), was ~0.53 mg/kg. All mutants except P52S were also used in the bioassay. Since the expression of P52S was in a trace amount, the obtained material was only used for tests on VGSCs in oocytes. Each purified mutant was injected into the mice through the tail vein at different doses to determine the LD50 value. These results are listed in Table 1. Mutants I6G, G43A, and L51G showed no detectable toxicity even at a dose of 25 mg/kg, which is ~47 times the LD50 of rBmK M1. Mutants G34A and G61A lost most of their toxicity (only ~2.5% of BmK M1). Mutants D3N, P9N, P9N/H10Y, and H10Y revealed a significantly higher potency resulting in a toxicity that was 150-300% of rBmK M1 (Table 1).

Effect of rBmK M1 and its mutants on VGSCs

Figure 4 shows the maximum efficacy of rBmK M1 on Na1.2/β1 (5 µM), Na1.5/β1 (2 µM), and para/tipE (500 nM) for comparison. Current traces recorded in the presence of rBmK M1 reveal that this toxin induces a slowing of the inactivation process. To clearly present the effect of the mutants on the three studied VGSCs, a topological way of presentation was chosen. The sites
where site-directed mutagenesis was performed are indicated on the ribbon structure of BmK M1 and shown per VGSC. Figure 5 displays the maximum efficacy of rBmK M1 and its mutants on Na\textsubscript{1.2}/β\textsubscript{1} (mammalian brain) at a concentration of 5 µM. This concentration was used for every mutant because the effect of BmK M1 itself on Na\textsubscript{1.2}/β\textsubscript{1} was not very pronounced. Figures 6 and 7 display the maximum efficacy of rBmK M1 and its mutants on Na\textsubscript{1.5}/β\textsubscript{1} (mammalian heart) and para/tipE (insect), respectively, shown at a concentration whereby the maximum effect was reached. This meant that concentrations up to 20 µM sometimes had to be applied to obtain a complete dataset from which the EC\textsubscript{50} values were derived.

The depicted current traces are the result of a two-electrode voltage clamp experiment on VGSCs expressed together with their β subunit (tipE for para) in Xenopus laevis oocytes. Current traces were evoked by a step depolarization ranging from -20 to 0 mV, depending on the studied VGSC, from a holding potential of -90 mV.

The slowing of inactivation induced by rBmK M1 and its mutants is concentration-dependent (see Table 2). The EC\textsubscript{50} values were determined according to previously published procedures (see materials and methods). EC\textsubscript{50} values of rBmK M1 and its mutants on Na\textsubscript{1.2}/β\textsubscript{1} were not determined, since rBmK M1 only had a small effect on this VGSC at high concentrations (5 µM). The EC\textsubscript{50} values on Na\textsubscript{1.5}/β\textsubscript{1} (A) are divided by the ones from para/tipE (B) and represented in the final column in Table 2. It is important to know that mutant values (A)/(B) <100 indicate an increased preference for Na\textsubscript{1.5}/β\textsubscript{1} (mammal). Mutually, (A)/(B) values >100 indicate an increased preference for para/tipE (insect). Asterisk in Table 2 indicates total insect specificity. A general overview of the results regarding efficacy and potency of rBmK M1 and its mutants on Na\textsubscript{1.2}/β\textsubscript{1}, Na\textsubscript{1.5}/β\textsubscript{1}, and para/tipE referenced to rBmK M1 is presented as a bar diagram in Fig. 8 (efficacy, upper part, and potency, lower part)

**Effects of the mutants on the vertebrate neuronal VGSC, Na\textsubscript{1.2}/β\textsubscript{1}**

As can be seen in Figs. 5 and 8, the following mutants displayed a remarkable increase in efficacy on Na\textsubscript{1.2}/β\textsubscript{1} with respect to rBmK M1: DeletionV1, R2A, D3A, D3N, P9N/H10Y, and H10Y. Mutants I6G, G43A, I57G, and P60A did not affect Na\textsubscript{1.2}/β\textsubscript{1} at concentrations up to 5 µM.

**Effects of the mutants on the vertebrate muscle VGSC, Na\textsubscript{1.5}/β\textsubscript{1}**

As can be seen in Figs. 6 and 8, several mutants caused a large increase in maximum efficacy on Na\textsubscript{1.5}/β\textsubscript{1}: DeletionV1, R2A, P60A, and P60G. Mutants D3N, G43A, L51G, and G61A have a diminished efficacy. The potencies of DeletionV1, R2A, Deletion1-3 (D3R), P9N/H10Y, G43A, L51G, I57G, P60A, and G61A on Na\textsubscript{1.5}/β\textsubscript{1} have decreased with respect to rBmK M1. Remarkably, the potency of H10Y on this VGSC has increased about sevenfold.

**Effects of the mutants on the insect VGSC, para/tipE**

From Figs. 7 and 8, it becomes obvious that all mutants possess about the same maximum efficacy on para/tipE as compared with rBmK M1. Nevertheless, large mutant-dependent differences in potency are noticeable. Mutants with a strong decrease in potency are R2A, I6G, P9N, G43A, and L51G.
DISCUSSION

In this study, 14 N- and C-terminal residues from the α-like toxin BmK M1 are analyzed by site-directed mutagenesis. Most of these residues are located in the region of the N- and C-terminal segment confirming previously published results indicating their importance for bioactivity (25). This “NC domain,” which is constituted by the five-residue-turn (residues 8-12) and the C-terminal region (residues 56-64), varies in amino acid composition and spatial arrangement among α-toxins, suggesting that its conformation is important for toxin function and specificity.

By correlating data from high impact substitutions such as glycine and differently charged residues (positive to negative and vice versa), partially based on sequence similarity with other insect and/or mammal specific scorpion toxins, our results clearly indicate that most of these residues are specifically involved in either structural stability or pharmacological function or both. To discriminate between effects resulting from structural alterations and those reflecting a pure pharmacological interaction with the VGSC receptor site, CD spectroscopy was used.

**Residues involved in structural stability (mutants with altered CD spectrum)**

The expression of the P52S mutant in a trace amount indicates that replacement of a proline at this position by a serine is hampering its folding. The low amount of obtained material was saved for electrophysiological experiments. Therefore, a CD spectrum and a bioassay on mice could not be performed. From Figs. 5–7, it seems that the position of this proline contributes to the final turn and orientation of the C terminus. The efficacy of this mutant remains unaltered, but the potency is reduced, in particular on para/tipE resulting in a less potent but more mammal-specific toxin.

The general consensus among the mutants of which the CD spectrum has been critically altered is a much lower potency in the bioassay on mice and on the individual VGSCs. The R2E, I6G (loss of hydrophobic side chain that is part of the conserved hydrophobic core) and G34A (disrupted β2-sheet) mutants have gained mammal specificity, but their potency has decreased dramatically (Tables 1 and 2; Fig. 8). The L51G (loss of hydrophobic side chain that is part of the conserved hydrophobic core) and G61A mutant seem to be more insect selective although their potency has decreased. The D3A mutant, protruding from the surface of the molecule, still has a high potency in the bioassay but the effect on Naᵥ1.5/β₁ in oocytes is severely diminished indicating an augmented insect-specificity. However, it is noteworthy that the efficacy of this mutant located in the first β-sheet on Naᵥ1.2/β₁ is greatly increased. This feature might explain the toxicity level in the bioassay. It can be hypothesized that substitution of the aforementioned residues causes profound structural changes and as a consequence the pharmacological properties of rBmK M1 are influenced.

**Residues involved in pharmacological function**

No structural perturbations were present in the following series of mutants since their CD spectra did not change with respect to rBmK M1. In general, the results of the bioassay on mice of the mutants are in concordance with the measurements on the VGSCs expressed in oocytes. A good marker for changes in selectivity toward mammals (Naᵥ1.2/β₁ and Naᵥ1.5/β₁) or insects (para/tipE) is presented in Table 2 (see also Results).
Mutants with no change in selectivity

P9N/H10Y, V59G, and P60G did not alter the selectivity of rBmK M1 toward mammals or insects. The EC$_{50}$ values of P9N/H10Y on Na$_{1.5}$/β$_1$ and para/tipE are decreased proportionally, so selectivity is not affected. In the bioassay, this mutant seems more potent than rBmK M1. A plausible explanation for this can be the highly improved efficacy on Na$_{1.2}$/β$_1$ (Fig. 8). The V59G and P60G mutants revealed a slight proportional decrease of EC$_{50}$ values on oocytes, which is in concordance with the bioassay. The P60G mutant is slightly more potent than rBmK M1 on mice what can be explained by the gain in efficacy on Na$_{1.5}$/β$_1$ of this mutant. P9N/H10Y, V59G, and P60G are located in the NC domain, but they only (slightly) affect the potency and efficacy of rBmK M1 on all tested VGSCs and there was no change in selectivity.

Mutants promoting mammal selectivity

Only one C-terminal mutant (P60A) causes rBmK M1 to become more mammal specific, although the efficacy on Na$_{1.2}$/β$_1$ has disappeared (Table 2). It has been previously reported that the specific orientation of the C-terminal segment mediated by the five-residue-turn is relevant to the preference of various α-toxin subgroups for phylogenetically distinct VGSCs receptor sites. This resulted in the conclusion that it is mainly the NC domain that confers selectivity for a certain VGSC (24).

According to Table 2, mutants R2A, D3N, Deletion1-3 (D3R), P9N, and H10Y have gained mammal-specificity without changing the overall structure of rBmK M1. Especially P9N (increased efficacy on Na$_{1.2}$/β$_1$, slightly increased potency on Na$_{1.5}$/β$_1$) and H10Y (substantial increase in efficacy on Na$_{1.2}$/β$_1$, sevenfold increase of potency on Na$_{1.5}$/β$_1$) have become highly specific for mammals. Their elevated toxicity toward mammals is also confirmed in the bioassay (Table 1). It is possible that the tyrosine at position 10 (in the NC domain) can form a more tightly packed NC domain by forming more hydrogen bonds and sustaining more hydrophobic interactions with the aliphatic residues from the C tail (e.g., ring-ring interaction with histidine at position 64). This mutation therefore results in a more hydrophobic character of the NC domain that reaches out for interaction with mammalian VGSCs. Since these mutations do not alter the general structure of rBmK M1, it does not support parts of a previous study where it was postulated that mammal-specific scorpion toxins lack the protruding shape of the NC domain (25). Both H10 and P60 have also been indicated in one of our previous studies to be evolutionary important (37). This study confirms their pharmacological relevance.

Mutants promoting insect selectivity

When measured on VGSCs, the deletion-V1 mutant seems to have gained insect selectivity. However, this mutant is still toxic for mice. This may be caused by its highly increased efficacy on Na$_{1.2}$/β$_1$ and Na$_{1.5}$/β$_1$. The valine at this position reaches out to the aspartic acid at position 53. Disturbing these neighbors obviously affects the potency of the toxin on Na$_{1.5}$/β$_1$. When the highly conserved glycine at position 43 is mutated to the aliphatic residue alanine (G43A), it is remarkable to see that rBmK M1 becomes totally insect specific (no activity on mice), although its potency is less than rBmK M1 itself. This residue is located next to Y42 in the loop between the β$_2$ and β$_3$ sheet, which is remarkably different in sequence and structure between α- and β-toxins (38). Apparently, this loop also contributes to the target specificity of toxin. As seen in
different crystal structures of scorpion α-toxins, this loop adopts various conformational states, indicating its inherent flexibility. Recent site-directed mutagenesis of BmK M1 identified that the Y42 residue is important for the pharmacological function of this toxin (23). Considering that G43 is adjacent to this residue, it is plausible to infer that the highly conserved G43 provides a certain degree of flexibility required in the functional performance of the Y42 residue. Therefore, this glycine residue should be considered as part of this functional site.

In conclusion, based on the mutagenesis results in this study, it seems plausible that the charge distributions or hydrophobicity of the protruding NC domain determines the target specificity of scorpion α-like toxins. Remarkably, one of the most surprising findings of our work is the fact that one point mutation (e.g., H10Y) may completely alter the target specificity of α-like scorpion toxins. One conclusion from a previously conducted extensive study of Karbat et al. (25) was that scorpion α-toxins active on insects still possess the protruding NC domain while the scorpion α-toxins active on mammals do not have this shape. Hence, it was stated that this phenomenon could be responsible for VGSC subtype selectivity. However, from our data it seems that it is not the presence of the protruding shape of the NC domain itself that causes the toxin to be specific for mammals or insects. In several of the mutants that have gained mammal specificity, the general structure of the toxin indeed did not significantly change. The presence of several functionally important epitopes is more likely to be the key in this issue. An important part of these toxin regions was identified in the same study from Karbat et al. (25).

However, as a result from our experiments, we propose a “fine-tuning” of this previous hypothesis stating that two domains, the NC and the core domain, determine the target specificity of a scorpion α-like toxin. We hypothesize that there are three epitopes (not two) that determine the target specificity of toxin: 1) the first three N-terminal residues, 2) the five-residue-turn (residues 8-12) in combination with the C tail (residues 57-61), and 3) the loop between the β2 and β3 sheet including the adjacent glycine in the β3 sheet (residues 40-43).

ACKNOWLEDGMENTS

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REFERENCES


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Table 1

Overview of expression levels and toxicity on mice and molecular mass of rBmK M1 and its mutants

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Expression</th>
<th>Toxicity (LD&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>Relative toxicity</th>
<th>Molecular Mass Estimated</th>
<th>Molecular Mass Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/liter</td>
<td>mg/kg</td>
<td>%</td>
<td>Da</td>
<td>Da</td>
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<tr>
<td>rBmK M1</td>
<td>≈3</td>
<td>0.53</td>
<td>100</td>
<td>7418.4</td>
<td>7418.0</td>
</tr>
<tr>
<td>Deletion1-3 (D3R)</td>
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<td>4.10</td>
<td>13</td>
<td>6847.7</td>
<td>6847.8</td>
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<tr>
<td>DeletionV1</td>
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<td>82</td>
<td>7319.3</td>
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<td>R2A</td>
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<td>R2E</td>
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<td>40</td>
<td>7391.1</td>
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</tr>
<tr>
<td>D3A</td>
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<td>78</td>
<td>7373.9</td>
<td>7375.8</td>
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<td>D3N</td>
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<td>143</td>
<td>7417.4</td>
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<td>&lt;2</td>
<td>7362.3</td>
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<td>P9N</td>
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<td>H10Y</td>
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<td>7444.4</td>
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<td>G34A</td>
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<td>2.6</td>
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<td>7432.0</td>
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<tr>
<td>G43A</td>
<td>1-2</td>
<td>&gt;25</td>
<td>&lt;2</td>
<td>7432.4</td>
<td>7433.0</td>
</tr>
<tr>
<td>L51G</td>
<td>1-2</td>
<td>&gt;25</td>
<td>&lt;2</td>
<td>7364.3</td>
<td>7377.0</td>
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<tr>
<td>P52S</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>7408.4</td>
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<td>G61A</td>
<td>1-2</td>
<td>18.58</td>
<td>2.8</td>
<td>7432.4</td>
<td>7433.7</td>
</tr>
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Table 2

Overview of the EC$_{50}$ values obtained on Na$_{1.5}$/$\beta_1$ (A) and para/tipE (B) expressed in *Xenopus laevis* oocytes

<table>
<thead>
<tr>
<th>Toxin/mutant</th>
<th>EC$<em>{50}$ Na$</em>{1.5}$/$\beta_1$ (A)</th>
<th>EC$_{50}$ para/tipE (B)</th>
<th>(A)/(B)$_{\text{norm}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type BmK M1</td>
<td>500 ± 30 nM</td>
<td>30 ± 6 nM</td>
<td>100</td>
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<tr>
<td>DeletionV1</td>
<td>4.3 ± 0.2 µM</td>
<td>75 ± 15 nM</td>
<td>343</td>
</tr>
<tr>
<td>R2A</td>
<td>7.6 ± 0.6 µM</td>
<td>1.3 ± 0.2 µM</td>
<td>35</td>
</tr>
<tr>
<td>R2E</td>
<td>787 ± 172 nM</td>
<td>670 ± 44 nM</td>
<td>7</td>
</tr>
<tr>
<td>D3A</td>
<td>8.4 ± 0.4 µM</td>
<td>226 ± 28 nM</td>
<td>223</td>
</tr>
<tr>
<td>D3N</td>
<td>362 ± 102 nM</td>
<td>117 ± 6 nM</td>
<td>19</td>
</tr>
<tr>
<td>Deletion1-3 (D3R)</td>
<td>1.6 ± 0.1 µM</td>
<td>515 ± 35 nM</td>
<td>19</td>
</tr>
<tr>
<td>I6G</td>
<td>1.2 ± 0.1 µM</td>
<td>2.1 ± 0.1 µM</td>
<td>4</td>
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<tr>
<td>P9N</td>
<td>374 ± 46 nM</td>
<td>723 ± 53 nM</td>
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<tr>
<td>P9N/H10Y</td>
<td>3.5 ± 0.1 µM</td>
<td>153 ± 15 nM</td>
<td>137</td>
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<td>H10Y</td>
<td>74 ± 15 nM</td>
<td>54 ± 6 nM</td>
<td>8</td>
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<tr>
<td>G34A</td>
<td>5.6 ± 0.7 µM</td>
<td>1.3 ± 0.2 µM</td>
<td>26</td>
</tr>
<tr>
<td>G43A</td>
<td>-</td>
<td>1.7 ± 0.1 µM</td>
<td>*</td>
</tr>
<tr>
<td>L51G</td>
<td>-</td>
<td>7.0 ± 0.5 µM</td>
<td>*</td>
</tr>
<tr>
<td>P52S</td>
<td>607 ± 55 nM</td>
<td>506 ± 93 nM</td>
<td>7</td>
</tr>
<tr>
<td>I57G</td>
<td>-</td>
<td>75 ± 12 nM</td>
<td>*</td>
</tr>
<tr>
<td>V59G</td>
<td>1.3 ± 0.3 µM</td>
<td>67 ± 17 nM</td>
<td>116</td>
</tr>
<tr>
<td>P60A</td>
<td>2.9 ± 0.7 µM</td>
<td>612 ± 39 nM</td>
<td>28</td>
</tr>
<tr>
<td>P60G</td>
<td>389 ± 36 nM</td>
<td>18 ± 9 nM</td>
<td>129</td>
</tr>
<tr>
<td>G61A</td>
<td>-</td>
<td>235 ± 34 nM</td>
<td>*</td>
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(A)/(B)$_{\text{norm}}$ for rBmK M1 is set to 100 as a reference. Values <100 indicate an increased preference for Na$_{1.5}$/$\beta_1$ and values >100 indicate para/tipE selectivity; $n \geq 3$. *Total insect selectivity.
Figure 1. Location of residues selected for mutagenesis on the amino acid sequence (A) and 3D structure (B) of BmK M1. Residues that were mutated are highlighted in gray-white (A). Amino acids selected for mutation are indicated with cyan-colored α-carbon atoms on 3D structure of BmK M1 (B). Some residues close to selected ones and involved in function are indicated in blue (24).
Figure 2. As an example, 2-step purification (A and B) and mass spectrum (C) for mutant H10Y are shown. A) Purification of H10Y on a CM32 cation-exchange column. CM32 column was eluted with a 3 step-gradient solution: 0.2 N, 0.3 N, 0.5 N NaCl in 0.1 N NaAc-Hac at pH 4.2. H10Y (*) was found in the 0.3 N NaCl fraction. B) Purification of H10Y by reversed-phase chromatography on a C18 column. C) Mass spectrum of H10Y.
Figure 3. CD spectrum of rBmK M1 and its mutants with deviant spectra. The measurement is carried out in UV range 250-200 nm on a Jasco 720 system at pH 8.0 with a concentration of 1.0 mg/ml at room temperature. All other mutants with spectra identical to rBmK M1 are not shown.
Figure 4. Representation of maximum effects of rBmK M1 on inactivation kinetics of Na\textsubscript{1.2}/β\textsubscript{1}, Na\textsubscript{1.5}/β\textsubscript{1}, and para/tipE. Current traces were evoked by depolarisations ranging from -20 to 0 mV, depending on VGSC, during 25 ms from a holding potential of -90 mV. *Control condition where no toxin was added. The following concentrations are shown: Na\textsubscript{1.2}/β\textsubscript{1}, 5 µM; Na\textsubscript{1.5}/β\textsubscript{1}, 2 µM and para/tipE, 500 nM. Scale y-axis represents 0.75 µA for Na\textsubscript{1.2}/β\textsubscript{1}, and para/tipE and 1.5 µA for Na\textsubscript{1.5}/β\textsubscript{1}. 
Figure 5. Effect of rBmK M1 mutants on inactivation kinetics of Na,1.2/β, channels (mammalian brain) expressed in *Xenopus laevis* oocytes. All concentrations shown are 5 µM. All mutant positions are indicated on the ribbon structure of BmK M1. Scale bar y-axis for all mutants is ~0.5 µA. Figure was produced using MOLMOL 2K.1 (40). The most rapidly inactivating currents represent control conditions.
Figure 6. Maximum effect of rBmK M1 mutants on the inactivation kinetics of Na,1.5/β,1 channels (mammalian heart) expressed in Xenopus laevis oocytes. Concentrations shown are: 1 µM for H10Y; 2 µM for P9N and P60G; 5 µM for D3N; 10 µM for DeletionV1, R2E, D3A, D3R (Deletion1–3), I6G, P52S, I57G, V59G, P60A, and G61A; 15 µM for R2A and P9N/H10Y; 20 µM for G34A, G43A, and L51G. All mutant positions are indicated on the ribbon structure of BmK M1. Scale bar y-axis for all mutants is ~1.5 μA. The most rapidly inactivating currents represent control conditions.
Figure 7. Maximum effect of rBmK M1 mutants on the inactivation kinetics of para/tipE channels (insect) expressed in Xenopus laevis oocytes. The following concentrations are shown: 200 nM for P60G; 500 nM for P9N/H10Y; 1 µM for DeletionV1, D3N, P52S, I57G, V59G, and G61A; 2 µM for R2E, D3A, D3R (Deletion1-3), P9N, H10Y, and P60A; 5 µM for R2A, I6G, G34A, and G43A; 20 µM for L51G. All mutant positions are indicated on the ribbon structure of BmK M1. Scale bar y-axis for all mutants is ~0.5 µA. The most rapidly inactivating currents represent control conditions.
Figure 8. Bar diagram representing maximum efficacies (**upper panel**) of studied mutants referenced to rBmK M1 (100) on Na,1.2/β1 (yellow), Na,1.5/β1 (red), and para/tipE (blue). **Lower panel** Obtained potencies after sigmoidal fit of the data of studied mutants on Na,1.5/β1 and para/tipE referenced to rBmK M1. All data are means ± SE of at least 3 measurements.