Chinese-scorpion (*Buthus martensi* Karsch) toxin BmK α IV, a novel modulator of sodium channels: from genomic organization to functional analysis

Zhi-Fang CHAI*, Mang-Mang ZHU*, Zhan-Tao BAI*, Tong LIU*, Miao TAN*, Xue-Yan PANG† and Yong-Hua JI*†¹

*Graduate School of the Chinese Academy of Sciences, Shanghai Institute of Physiology, Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, People's Republic of China, and †School of Life Sciences of Shanghai University, Shanghai 200444, People's Republic of China

In the present study, BmK α IV, a novel modulator of sodium channels, was cloned from venomous glands of the Chinese scorpion (*Buthus martensi* Karsch) and expressed successfully in *Escherichia coli*. The BmK α IV gene is composed of two exons separated by a 503 bp intron. The mature polypeptide contains 66 amino acids. BmK α IV has potent toxicity in mice and cockroaches. Surface-plasmon-resonance analysis found that BmK α IV could bind to both rat cerebrocortical synaptosomes and cockroach neuronal membranes, and shared similar binding sites on sodium channels with classical AaH II (α -mammal neurotoxin from the scorpion *Androctonus australis* Hector), BmK AS (β -like neurotoxin), BmK IT2 (the depressant insect-selective neurotoxin) and BmK abT (transitional neurotoxin), but

not with BmK I (α -like neurotoxin). Two-electrode voltage clamp recordings on rNav1.2 channels expressed in *Xenopus laevis* oocytes revealed that BmK α IV increased the peak amplitude and prolonged the inactivation phase of Na⁺ currents. The structural and pharmacological properties compared with those of other scorpion α -toxins suggests that BmK α IV represents a novel subgroup or functional hybrid of α -toxins and might be an evolutionary intermediate neurotoxin for α -toxins.

Key words: *Buthus martensi* (Chinese scorpion) α -like neurotoxin IV (BmK α IV), cockroach neuronal membrane, rat brain II sodium channel α -subunit (rNav1.2), rat cerebrocortical synaptosome.

INTRODUCTION

VGSCs (voltage-gated sodium channels) are critical for the initiation and propagation of action potential in excitable cells and are related to pain and a series of channel diseases such as epilepsy [1]. They are composed of a pore-forming α -subunit associated with up to four auxiliary β -subunits [1,2]. To date, seven topologically distinct receptor sites for neurotoxins on the α -subunit of VGSCs have been identified, all of which are linked to specific effects on channel function. Numerous natural neurotoxins targeted to VGSCs by affecting either gating properties or ion permeation of VGSCs, have been used extensively for identification and characterization of the subtypes of sodium channels. Among them, scorpion neurotoxins are divided into α - and β -classes according to their mode of actions and binding properties to VGSCs [3,4].

Scorpion α -neurotoxins mainly slow the inactivation of VGSCs and consequently prolong the action potential upon binding to receptor site 3, and are classified into three major groups based on the preferential toxicity to mammals or insects and their differential binding properties. Classical α -mammal neurotoxins bind with high affinity to rat brain VGSCs and are highly toxic to mammals, while they are practically non-toxic to insects. α -Insect neurotoxins bind to insect VGSCs with high affinity and are very active in insects but less potent in mammals. α -Like neurotoxins, which could not bind to rat brain synaptosomes, are active in both mammal and insect nervous systems [3]. Scorpion

β -neurotoxins modify the voltage dependence of activation of sodium channels by binding to receptor site 4 of VGSCs. According to their pharmacological activities, the β -neurotoxins can be divided into several groups: β -mammal toxins; β -toxins active on both mammals and insects; excitatory insect-selective toxins; and depressant insect-selective toxins and β -like toxin [3,4].

The Chinese scorpion BmK (*Buthus martensi* Karsch), of the family Buthidae, is considered to possess a moderately active venom [5]. Many kinds of long-chain scorpion neurotoxins modulating the pharmacological properties of VGSCs have been purified from BmK venom [6]. Moreover, it has been demonstrated that the efficacy of BmK venom in treating pain and epilepsy depends on the different kinds of these modulators of sodium channels from scorpion BmK venom [7–11]. Therefore elucidation of the inherent functional and structural interaction between scorpion neurotoxins and sodium channels may provide a promising perspective for the future design of selective drugs. On the other hand, adaptive evolution of scorpion neurotoxins and ion channels have also been constructed on the basis of genomic organization, structure and pharmacology of toxin and ion channels, as well as scorpion species distribution. The difference of function and components in scorpion venom might reflect the divergence of scorpion species status and distribution [4,12,13].

In the present paper we report that BmK α IV, a novel sodium channel modulator, was cloned and expressed heterogeneously,

Abbreviations used: AaH II, α -mammal neurotoxin from *Androctonus australis* Hector (Sahara scorpion); Amm, *Androctonus mauretanicus mauretanicus* (black scorpion); BmK, *Buthus martensi* Karsch (Chinese scorpion); BmK abT, transitional neurotoxin; BmK AS, β -like neurotoxin; BmK I, α -like neurotoxin; BmK IT2, depressant insect-selective neurotoxin; i.c.v., intracerebroventricularly; Lqh, *Leiurus quinquestriatus hebraeus* (Hebraei scorpion); Lqq, *Leiurus quinquestriatus quinquestriatus* (death-stalker scorpion); RACE, rapid amplification of cDNA ends; rNav1.2, rat brain II sodium channel α -subunit; RU, resonance unit(s); VGSC, voltage-gated sodium channel; UPM, universal primer mix.

¹ To whom correspondence should be addressed (email yhj@server.shnc.ac.cn or yhj@staff.shu.edu.cn).

The nucleotide sequence data reported have been deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number DQ058408.

and its pharmacological and electrophysiological characteristics on sodium channels were investigated.

MATERIALS AND METHODS

Materials

Escherichia coli strains DH5 α and BL21(DE3) were used for plasmid amplification and protein expression respectively. pET-28a(+) was used as the vector for protein expression. Adult male Sprague–Dawley rats and Kunming mice were provided by the Shanghai Center for Experimental Animals, Shanghai, China. Male adult *Periplaneta americana* and *Blattella germanica* cockroaches were from the Shanghai Institute of Entomology (Chinese Academy of Sciences, Shanghai, People's Republic of China). Female *Xenopus laevis* were purchased from Chinese Academy of Sciences. BmK I (α -like neurotoxin), BmK abT (transitional neurotoxin), BmK AS (β -like neurotoxin) and BmK IT2 (depressant insect-selective neurotoxin) were purified according to procedures published previously [14–17]. AaH II (α -mammal neurotoxin from the scorpion *Androctonus australis* Hector) was generously given by Professor Hervé Rochat (Laboratoire de Biochimie, Faculté de Médecine Secteur Nord, Marseille, France) and Dr Marie-France Martin-Eauclaire (UMR 6560 CNRS, Université de la Méditerranée, Institut Jean Roche, Faculté de Médecine Nord, Marseille, France). The alkaloid veratridine was from Sigma. All other reagents used were of analytical grade. All animal protocols followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animals, Chinese Academy of Sciences.

Cloning of the BmK α IV gene

Total RNA was extracted using TRIzol[®] reagent (Invitrogen) from 2 g of venomous glands of BmK (collected in Henan Province, China). The BmK α IV gene was cloned by 5'- and 3'-RACE (rapid amplification of cDNA ends) using SMART[™] RACE cDNA Amplification Kit (BD Biosciences Clontech). Primer 1 was designed as 5'-TTATGATACATTCCATCTTA-3' according to a conserved fragment of the 3' untranslated regions of two α -neurotoxins (BmK α 1 and AaH II) [18,19]. The PCR product was amplified using primer 1 and the UPM (universal primer mix) for 5'-RACE. Primer 2 was 5'-CTTTCCTCA-GAAAATTCCGTAAAACGGTTC-3' corresponding to the 5' untranslated regions of BmK α IV determined by 5'-RACE. The PCR products were amplified using primer 2 and UPM primer for 3'-RACE. Genomic DNA was isolated from venomous glands of BmK using the Wizard Genomic DNA Purification Kit (Promega). The PCR products were amplified using primer 1 and primer 2 and ligated into pGEM-T vector (Promega), then transformed into *E. coli* DH5 α . The plasmid was isolated and then subjected to DNA sequencing.

Construction of expression vector pET-BmK α IV

Two synthetic oligonucleotide primers were designed for amplifying the encoding region of BmK α IV. The forward primer was 5'-TGGAGTTAACATATGGTACGCGATGGTTATATTG-3', corresponding to the cDNA sequence of the N-terminal residues (1–6) of BmK α IV and with the restriction site of NdeI is underlined. The reverse primer was 5'-GTGCACTGGATCCTT-AACCGCCATTGCATCTTCC-3', corresponding to the cDNA sequence of the C-terminal residues (61–66) of BmK α IV and with the BamHI restriction site underlined. Both primers were used in a PCR with cDNA and the product was digested with NdeI and BamHI. The digested product was subcloned into

the corresponding NdeI and BamHI restriction sites so that the product was in-frame with the His₆ tag of the pET-28a(+) vector (Novagen). This construct thus allowed the expression of a His₆–BmK α IV fusion protein. When required, the fusion protein was cleaved with the protease thrombin (Amersham Biosciences) according to the manufacturer's protocol.

Expression and purification of recombinant BmK α IV

The expression plasmid pET-28a(+)-BmK α IV was transformed into *E. coli* BL21(DE3) cells (Invitrogen). *E. coli* was grown at 37°C overnight in Luria–Bertani broth containing 40 μ g/ml kanamycin. Then isopropyl β -D-thiogalactopyranoside to a final concentration of 0.2 mM was added when the culture reached an attenuation (D_{600}) of 0.4–0.6 and the cells were grown for a further 16 h at 22°C. Cells from 1 litre of culture medium were harvested by centrifugation at 5000 g for 6 min, then resuspended in lysis buffer (30 mM Tris/HCl, 2.5 mM EDTA, pH 8.0, 0.5 mM PMSF and 10 μ g/ml lysozyme), and sonicated on ice for 20 min (four bursts/min). The insoluble pellet obtained by centrifugation (12 000 g, 15 min) was rinsed three times with washing buffer [25% (w/v) sucrose, 5 mM EDTA and 1% (v/v) Triton X-100 in PBS]. Then the fusion proteins in the pellets were purified by affinity chromatography on Ni²⁺-nitrilotriacetate–agarose beads (Qiagen) under the denaturing conditions according to the manufacturer's instructions. Protein renaturation was initiated by 50-fold dilution in 0.2 M ammonium acetate (pH 8.0). The dialysis buffer was changed every 24 h three times in total at 22°C. After renaturation, the insoluble proteins were removed by centrifugation (12 000 g, 15 min), while the soluble fusion proteins were concentrated by ultrafiltration membranes (3 kDa molecular-mass cut-off; Amicon) and purified by reverse-phase HPLC (Develosil; ODS-HG-5; 25 cm $\times$ 0.5 cm; Nomura Chemical Co.). Mobile phases were solvent A [saturated HCl/water, 1:999, (v/v)] and solvent B [60% (v/v) acetonitrile], and elution was performed with two successive linear gradients: 0–15% of solvent B for 5 min, followed by 15–80% of solvent B for the next 25 min at a flow rate of 1 ml $\cdot$ min⁻¹. Fractions were hand-collected by monitoring the effluent signal at 280 nm and dried in a vacuum centrifuge. Immunoblotting was carried out with anti-(His tag) antibody (Santa Cruz Biotechnology). The proteins were detected by ECL[®] (enhanced chemiluminescence; Amersham Biosciences). The fusion proteins were cleaved with 10 units of thrombin/mg of protein substrate (Amersham Biosciences) in buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl and 2.5 mM CaCl₂) for 16 h at 22°C, and purified by reverse-phase HPLC. The molecular mass of the purified products was determined by a REFLEX III matrix-assisted laser-desorption ionization–time-of-flight mass spectrometer (Bruker-Franzen Analytik).

CD spectroscopy and N-terminal sequencing

CD spectroscopy of BmK α IV (0.2 mg/ml in 20 mM sodium phosphate buffer, pH 7.0) was carried out on a J-810 system (JASCO) at room temperature (21–23°C). The spectrum was recorded from 190 to 250 nm at a scan rate of 100 nm/min with a time constant of 1 s on four occasions. N-terminal chemical protein sequencing was performed on PROCISE[™]492cLC Protein Sequencing System (Applied Biosystems).

Toxicity assay

Toxicity assays on mice were performed as described by Ji et al. [14]. Kunming mice (20 $\pm$ 2 g) were briefly anaesthetized with diethyl ether and injected i.c.v. (intracerebroventricularly) with five doses of BmK α IV (0.05, 0.15, 0.25, 0.35 and 0.5 μ g in

2 μ l of saline) or control (2 μ l of saline). Eight mice were used for each dose. Then the mice were placed in a 40 cm $\times$ 30 cm $\times$ 50 cm transparent glass box for continuous monitoring of the symptoms of neurotoxicity during the first 1 h post-injection or until death. The LD₅₀ for BmK α IV was estimated 24 h after injection. The cockroach was employed for the insect toxicity assay as described previously [20,15]. Different concentrations of BmK α IV (0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 μ g, dissolved in 2 μ l of 0.15 M NaCl containing 1 mg/ml BSA) were injected into the abdominal segment of male *B. germanica* cockroaches (80–100 mg, n = 8 for each dose). The control group received 2 μ l of vehicle solution without BmK α IV (n = 8). The toxicity was monitored for 48 h after injection of BmK α IV. LD₅₀ values were calculated according to the method developed by Reed and Muench [21].

Neuronal synaptosomes preparation

Rat cerebrocortical synaptosomes were prepared from adult male Sprague–Dawley rats (250 $\pm$ 10 g) according to the previous description [22]. Insect neuronal membrane of nerve cord was prepared from male adult cockroach *P. americana* (700 $\pm$ 50 mg) following the procedure described previously [23]. Both types of synaptosomes were resuspended in loading buffer (140 mM choline chloride, 1.8 mM CaCl₂, 5.4 mM KCl, 0.8 mM MgSO₄, 10 mM D-glucose and 25 mM Hepes, pH 7.4) and used for the biosensor assay at once. A combination of proteinase inhibitors consisting of 50 μ g/ml PMSF, 1 μ M pepstatin A, 1 mM iodoacetamide and 1 mM 1,10-phenanthroline was added to all buffers used in the procedure. The concentration of the synaptosome suspension was determined using a Bio-Rad protein assay with BSA as a standard.

Binding analysis

The binding assays were performed using surface plasmon resonance using BIAcore 3000 biosensor systems (Amersham Biosciences) at room temperature. BmK α IV (40 μ l of 40 μ g/ml in 10 mM acetate buffer, pH 5.0) was immobilized on the surface of the carboxymethylated dextran chip (CM5) using the Amine Coupling Kit according to the manufacturer's instructions (Amersham Biosciences). The control channel on the same sensor chip was processed according to the protocol described above but without peptide couple. Rat cerebrocortical synaptosomes and cockroach nerve-cord neuronal membranes dissolved in the loading buffer were passed over the chip surface. The procedure was carried out in a running buffer of Hepes-buffered saline (150 mM NaCl, 3.5 mM EDTA, 0.05 % BIAcore surfactant P-20 and 10 mM Hepes, pH 7.4) at a flow rate of 5 μ l/min. The regeneration of the chip surface was accomplished by the injection of 10 mM NaOH. The results were fitted and analysed using BIAevaluation 3.1 software.

Two-electrode voltage clamp recording in *X. laevis* oocytes

cRNAs encoding *rNav1.2* (rat brain II sodium channel α -subunit; kindly given by Professor Alan L. Goldin, Department of Microbiology and Molecular Genetics, University of California Irvine, Irvine, CA, U.S.A.) were transcribed *in vitro* with T7 RNA polymerase mMESSAGE mMACHINE[®] transcription kit (Ambion). Oocytes were removed from adult female *X. laevis* and incubated with 1 mg/ml type IA collagenase (Sigma) in an OR2 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂ and 5 mM Hepes, pH 7.5) for about 3 h. After washing, the isolated oocytes were transferred into ND-96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM Hepes, pH 7.5) supplemented with 0.1 mg/ml gentamicin, and the healthy oocytes in stage V–VI were chosen to be injected (1 ng/oocyte)

with *rNav1.2* cRNAs. Both injected and non-injected oocytes were further incubated for 40 h at 20 °C in ND-96 medium.

Na⁺ currents were recorded using two-electrode voltage clamps with the TURBO TEC-03X, INT-20 and Cellwork E 5.5 software (NPI Electronic, Tamm, Germany) at room temperature. The sensitivity of current output was 1 V/ μ A, and the filter was chosen at 2 kHz. Na⁺ current amplitudes were controlled between 5000 and 20000 nA. Data acquisition and analysis were performed using the Cellworks Reader 3.6 (NPI Electronic) and Origin 7.0 (OriginLab Corp., Northampton, MA, U.S.A.).

Sequence analysis and molecular modelling

Amino acid sequences of representative α -toxins were obtained from the Swiss-Prot database (<http://www.expasy.org/sprot>) and aligned using ClustalX 1.81 (<http://bips.u-strasbg.fr/en/Documentation/ClustalX/>). A bootstrap consensus tree was generated using the PHYLIP package (http://www.genebee.msu.su/services/phree_reduced.html).

The three-dimensional homology modelling of BmK α IV was performed by the program MODELLER [24] running on an SGI O2 workstation. The three-dimensional structure of AaH II experimentally resolved at 1.3 Å (1 Å = 0.1 nm; Protein Data Bank accession no. 1PTX) was used as a template, it having close sequence identity with BmK α IV (68.2 %). The models were screened with the PROCHECK program [25], from which the best model was selected. The electrostatic calculations were done by using the GRASP software [26] running on an SGI O2 Workstation.

Statistical analysis

Results are presented as means $\pm$ S.E.M. and submitted to one-way ANOVA. The Dunnett test was employed to compare the control group with the different treatment groups. P < 0.05 was considered to be significant.

RESULTS

Genomic organization of BmK α IV

A full-length sequence of BmK α IV genomic DNA (GenBank[®] accession no. DQ058408) was cloned and found to be 896 bp in length. As shown in Figure 1, it consisted of two exons disrupted by a single intron of 503 bp. The intron was arranged between the first two bases of the glycine codon (the glycine residue is situated four residues upstream from the start of the mature peptide). The 5' and 3' splice sites were GTAAGATTTA and CTGACTACAG respectively. The intron of BmK α IV was AT-rich (62.0 %). The initiation codon (ATG) was at the +70 position, relative to the transcription start site, and the stop codon (TAA) was at the +828 position. The poly(A) signal (AATAAA) was located 47 bp downstream from the stop codon. The open-reading-frame region encoded BmK α IV precursor polypeptide with 85 amino acid residues which consisted of signal peptide (19 residues) and mature peptide named BmK α IV (66 residues).

Expression and purification of recombinant BmK α IV

His₆–BmK α IV was found predominantly in inclusion bodies when expressed in *E. coli*. The identification of His₆–BmK α IV was processed by immunochemical recognition by an anti-(His tag) antibody (Figure 2B). The recombinant BmK α IV was gained by thrombin cleavage from His₆–BmK α IV detected by Coomassie Blue staining of tricine/polyacrylamide gel (Figure 2A). The molecular mass (7733.59 Da) of BmK α IV determined by MS (Figure 2C) was consistent with the calculated value of the amino acid sequence deduced from the cDNA nucleotide

Figure 1 Nucleotide sequence of genomic DNA encoding BmK α IV

The intron is shown in lower-case letters. Primers 1 and 2 (underlined) designed for 5'-RACE and 3'-RACE respectively, were for genomic DNA amplification. The putative mature peptide (bold face), signal peptide (italic) and stop codon (asterisks) are indicated. The deduced amino acids are given below the nucleotide sequence. The polyadenylation signal (AATAAA) is double-underlined. The N-terminal partial amino acid sequence (eight residues) was determined by Edman degradation.

Figure 2 Identification of BmK α IV

(A) The expression and thrombin digestion of His₆-BmK α IV. Lane M, molecular-mass standards (kDa); lane 1, His₆-BmK α IV of the precipitated fractions purified from Ni²⁺-nitrilotriacetate column; lane 2, His₆-BmK α IV after renaturation; lane 3, thrombin cleavage of His₆-BmK α IV. (B) Western blotting for His₆ tag of the fusion protein. Lanes 1–3 correspond to those in (A). (C) Mass spectrum of BmK α IV. (D) CD spectrum of BmK α IV.

sequence and four extra amino acids resulting from the thrombin cleavage site and the restriction site using for cloning (7734.84 Da). The largest yield of bioactive products was about 2 mg/l of culture medium. The recombinant BmK α IV, hereafter just called 'BmK α IV', was used for the following experiments. The N-terminal sequence of BmK α IV was (Gly-Ser-His-Met)-Val-Arg-Asp-Gly-Tyr-Ile-Ala-Asp determined by Edman degradation (Figure 1), in which the first two residues (Gly-Ser) was the rest of the thrombin recognition site, and the following two (His-Met) were encoded by restriction-enzyme-site sequences. The secondary structure of the recombinant BmK α IV, determined by CD spectroscopy, showed it to be folded as a well-structured neurotoxin (Figure 2D).

Toxicity assay of BmK α IV

Excitatory neurotoxicity in mice, such as spasms, convulsions, rapid movements and death, appeared within 10–20 min after

Figure 3 Representative sensorgrams showing the kinetics of BmK α IV binding to rat cerebrocortical synaptosomes (A) and cockroach neuronal membranes (B)

BmK α IV was immobilized on a CM5 sensor chip [about 2100 RU (resonance units); 1 RU is equivalent to 1 pg/mm² of sensor surface] using procedures described in the Materials and methods section. Serial dilutions of neuronal membranes (20 μ l) of rat cerebrocortical synaptosomes or cockroach neuronal membranes were injected over the immobilized BmK α IV for 240 s (0–240) then dissociation was followed for 240 s (240–480). Non-specific binding was subtracted. Kinetic dissociation constants (k_{off}) were calculated for each curve in the dissociation phase (260–380 s) with BIAevaluation 3.1 software. The results are from the triplicate experiments. All procedures were performed at room temperature.

injection of BmK α IV. The LD₅₀ of BmK α IV by i.c.v injection to mice was estimated to be about 208 ng/20 g of body weight. The maximal dose and LD₅₀ of BmK α IV in cockroaches were determined to be 2.0 and 0.47 μ g/100 mg of cockroach body weight respectively at 6 h post-injection. During the 6 h post-injection, the symptoms of cockroach paralysis appeared within 15 min after injection of BmK α IV. At the lower dose of BmK α IV (0.05, 0.1 or 0.2 μ g), some of cockroaches were able to recover from paralysis, whereas at the higher dose of 0.5 or 1.0 μ g, there was little recovery. The mortality was increased gradually within 12–48 h of the application of BmK α IV at 0.5 or 1.0 μ g dose. No toxic responses or death occurred in the control group of mice or cockroaches.

Rat-cerebrocortical-synaptosome- and cockroach-neuronal-membrane-binding properties of BmK α IV

BmK α IV could bind to rat cerebrocortical synaptosomes and cockroach neuronal membranes in a concentration-dependent manner, but could not be dissociated from its binding site completely after elution with running buffer. The binding of BmK α IV to rat cerebrocortical synaptosomes was increased progressively during the association phase of 240 s. The dissociation happened rapidly at first and then became slower later (Figure 3A). However, BmK α IV bound quickly to cockroach neuronal membranes and then reached saturation gradually. The dissociation of BmK α IV from cockroach neuronal membranes was a slow process (Figure 3B). The kinetic dissociation constant (k_{off}) was calculated to be $(1.71 \pm 0.13) \times 10^{-3} \text{ s}^{-1}$ to rat cerebrocortical synaptosomes

Figure 4 Competitive experiments between BmK α IV and neurotoxins

Competition for BmK α IV binding to rat cerebrocortical synaptosomes ($375 \mu\text{g/ml}$) (A) and cockroach neuronal membranes ($50 \mu\text{g/ml}$) (B) by high concentrations of BmK α IV and native neurotoxins. Neuronal membranes were pre-incubated in the absence (control) or presence of different neurotoxins for 30 min at room temperature. Binding levels were recorded at 240 s. Non-specific binding was determined according to the RU produced in the absence of polypeptide immobilized on to the channel of CM5 sensor chip and this value was subtracted from the data. The RU value of the same concentration of synaptosomes through the sensor chip was used as the positive control. The percentage competition of binding by the competitor peptide was calculated according to the equation:

$$\text{Percentage competition of binding} = 100 \times (\text{RU in the presence of competitor peptide} / \text{RU in the absence of competitor peptide})$$

The percentage of the binding response was averaged and presented as the mean $\pm$ S.E.M. ($n = 3$; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ compared with control).

Figure 5 Voltage-dependent binding of BmK α IV to rat cerebrocortical synaptosomes (A) and cockroach neuronal membranes (B)

Rat cerebrocortical synaptosomes ($375 \mu\text{g/ml}$) or cockroach neuronal membranes ($50 \mu\text{g/ml}$) were pre-incubated in the absence (control) or presence of various concentrations of KCl for 30 min at room temperature. The binding levels were recorded at 240 s in the dissociation phase. The non-specific binding was subtracted and the binding response (in RU) was averaged:

$$\text{Percentage of control} = 100 \times (\text{RU at the different KCl concentration} / \text{RU in the control KCl concentration})$$

The values are means $\pm$ S.E.M. ($n = 3$; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$, compared with control).

and $(4.16 \pm 0.15) \times 10^{-3} \text{ s}^{-1}$ to cockroach neuronal membranes during 260–380 s respectively. The other kinetic parameters could not be calculated, since it was difficult to determine the molarity of target protein in synaptosomes with any certainty.

The results of the competitive assay of BmK α IV binding to rat cerebrocortical synaptosomes and cockroach neuronal membranes is shown in Figures 4(A) and Figure 4(B) respectively. The binding of BmK α IV could be inhibited by BmK α IV itself and potently by AaH II, even at quite low concentration (10^{-10} M). The inhibition of AaH II to BmK α IV binding on rat cerebrocortical synaptosomes ($70.1 \pm 2.0\%$) was stronger than that on cockroach neuronal membranes ($10.9 \pm 1.8\%$). Veratridine (10^{-4} M) could partially inhibit the binding of BmK α IV to rat cerebrocortical synaptosome ($73.4 \pm 2.8\%$), but not to cockroach neuronal membranes. Furthermore, the binding of BmK α IV to rat cerebrocortical synaptosome and cockroach neuronal membranes could be completely blocked by BmK abT and BmK AS. The competitive effect of BmK IT2 on the binding of BmK α IV to rat cerebrocortical synaptosomes was about $81.2 \pm 2.0\%$, whereas to the cockroach neuronal membranes it was complete. However, BmK I, an α -like neurotoxin, was unexpectedly found not to inhibit BmK α IV binding to either rat cerebrocortical synaptosomes or cockroach neuronal membranes.

Additionally, the binding of BmK α IV to rat cerebrocortical synaptosomes was voltage-dependent, but this was not the case for binding of the toxin to cockroach neuronal membranes, as shown in Figures 5(A) and Figure 5(B) respectively.

Electrophysiological analysis of the effect of BmK α IV on rNav1.2

Xenopus oocytes with rNav1.2 cRNA injection were held at -80 mV and depolarized by step pulses ranging from -80 mV to $+60$ mV for 600 ms in 10 mV increments. During BmK α IV application, the transient Na^+ currents were increased in a dose-dependent manner, and this effect could not be diminished completely by washing (Figure 6A). Moreover, the dynamic course of inactivation was slowed by BmK α IV at 100 nM, and the current component of ultra-slow inactivation was enhanced significantly (Figure 6C). The degree of ultra-slow inactivation was measured with the amplitudes of steady-state activated Na^+ currents, which were determined as the averages during the last 10 ms of the pulse steps. As a result, the time constant of dynamic inactivation was increased after 100 nM BmK α IV application (Figure 6D). The steady-state Na^+ currents were increased by about 70% ($P = 0.015$; Figure 6E) with 100 nM BmK α IV. Neither the voltage-dependence of activation (Figure 6B) nor that

Figure 6 Electrophysiological effect of BmK αIV on rNav1.2 channel expressed in *Xenopus* oocytes

Na⁺ currents in rNav1.2 were evoked by steps from −80 mV to +60 mV with increment of 10 mV, from a holding potential of −80 mV. (A) The relative peak Na⁺ currents in the absence (control) or presence of 10, 100 and 500 nM BmK αIV. BmK αIV application at 100 nM and 500 nM could significantly increase the amplitude of peak Na⁺ currents. (B) The current–voltage relationships for the peak Na⁺ currents in the absence (control) or presence of 100 nM BmK αIV application. (C) Representative Na⁺ currents in the absence (control) or presence of 100 nM BmK αIV application. (D) Time constant of dynamic inactivation (Tau) of rNav1.2 currents modulated by BmK αIV under control conditions (bottom) and after toxin application (top). (E) Statistical values of the steady-state Na⁺ currents after addition of 100 nM BmK αIV. Values are means ± S.E.M. (n = 6); *P < 0.05; **P < 0.01 compared with control. In (A), (B) and (E) the parameter on the ordinate is a unitless ratio.

	10	20	30	40	50	60	Identity
BmK αIV	VRDGYIADDKN-CAYFCGR-N-AYCDDCECKKKGAESGYCQWAGVYGNACWCYKLPDK---	VRIRVPG-RCNGG-					100.0%
BuK αTx	VRDGYIADDKN-CAYFCGR-N-AYCDEECIINGAESGYCQWAGVYGNACWCYKLPDK---	VRIRVSG-EC---					84.8%
α-mammal toxins							
Amm V	LKDGYIIDDLN-CTFFCGR-N-AYCDDCECKKKGGESGYCQWASPYGNACWCYKLPDR---	VSIEKEG-RCN---					75.8%
Lqq V	LKDGYIIDDKN-CTFFCGR-N-AYCDDCECKKKGGESGYCQWASPYGNACWCYKLPDR---	VSIEKEG-RCN---					75.8%
AaH II	VRDGYIIDDVN-CTYFCGR-N-AYCNEECLKKGESGYCQWASPYGNACWCYKLPDR---	VRTKGP-RCR---					68.2%
Lqh II	IKDGYIIDDVN-CTYFCGR-N-AYCNEECLKKGESGYCQWASPYGNACWCYKLPDR---	VRTKGP-RCR---					66.7%
CsE V	KKDGYPVDSGN-CKYECLEDD--YCNDELCLERKADKGYCYWGR---	VSCYCYGLPDN--SPTKTSG-KCNPA-					42.4%
Ts IV-5	KKDGYPVEYDN-CAYICWYNDNAYCDKLCDDKADSGYCYVWH---	ILCYCYGLPDN--SEPTKTSG-KCKS-					45.6%
Ts V	KKDGYPVEYDN-CAFAFCGYNDNAYCDKLCDDKADSGYCYVWS---	PDCYCYGLPEHILKEPTKTSG-RC-					42.3%
KurTx	KIDGYPVDYWN-CKRICWYNN-KYCNDLCKGLKADSGYCWGWT---	LSCYCYGLPDN--ARIKRS-RCRA-					40.3%
α-insect toxins							
Lqh aIT	VRDAYIAKNYN-CVYECFR-D-AYCNELCTKNGASSGYCQWAGKYGNACWCYALPDN---	VRIRVPG-KCHRK-					68.2%
Lqq III	VRDAYIAKNYN-CVYECFR-D-SYCNELCTKNGASSGYCQWAGKYGNACWCYALPDN---	VRIRVPG-KCH---					68.2%
α-like toxins							
BmK I	VRDAYIAKPHN-CVYECAR-N-EYCNDLCTKNGAKSGYCYWVGKYGNGWCIELPDN---	VRIRVPG-KCH---					65.2%
Bot I	GRDAYIAOPEN-CVYECAR-N-SYCNELCTKNGATSGYCYWVGKYGNGWCIELPDN---	VRIRVPG-KCH---					62.0%
Bom III	GRDAYIAOPEN-CVYHCFPGS-SGCDTLCKEKGATSGHCGFLPGSGVACWCDNLPNK---	VRIRVPG-KCH---					62.1%
Lqh III	VRDGYIAOPEN-CVYHCFPGS-SGCDTLCKEKGATSGHCGFLPGSGVACWCDNLPNK---	VRIRVPG-KCH---					48.5%
β-toxins							
BmK AS	-DNGYLLDKYTGCWKVCVNN-ESCNSECKIRGGYGYCYFWK---	LACFCQGARKS---					26.1%
BmK abT	KKSGYPTDHEG-CKNWCVLNH--SCGILCEGYGG-SGYCYFWK---	LACWCDIHN--WVPTWSRATNKRA-					25.0%
BmK IT2	--DGYIKGSG-CRVACLIGN-QGCLDKCRAVGSYGYCWTWG---	LACWCEGLPDN--KTWKSESNTCG--					34.3%

Figure 7 Alignment of the amino acid sequence of BmK αIV with the partially known sequences of α- and β-scorpion toxins

Sequences were aligned by using the ALIGN program of the Vector NTI package. The important regions are shaded in dark grey. Percentage identity is shown at the right of each sequence. Amm V was from *A. m. mauretanicus*, Lqq V and Lqq III from *L. q. quinquestriatus*, AaH II from *A. australis* Hector; Lqh II, Lqh III and Lqh αIT from *L. q. hebraeus*, KurTx from *Parabuthus transvaalicus* (Transvaal thick-tailed scorpion), CsE V from *Centruroides sculpturatus* (Arizona scorpion), Ts IV-5 and TS V from *Tityus serrulatus* (fat-tailed scorpion), Bot I from *Buthus occitanus tunetanus* (North-African scorpion), Bom III from *B. o. mardochei* (Moroccan scorpion) and Bukatx (bukatoxin), BmK I, BmK IT2, BmK AS and BmK abT from *B. martensi* Karsch.

of the steady-state inactivation (results not shown) was affected significantly by BmK αIV.

Alignment of BmK αIV with known scorpion α-neurotoxins

In Figure 7, the sequence comparison of BmK αIV with the partially known scorpion toxin sequences showed that BmK αIV shared high sequence similarity with bukatoxin (84.8%, α-toxin from BmK) and classical α-mammal neurotoxins, such as Lqq V (*Leiurus quinquestriatus quinquestriatus* V)/Amm V (*Androctonus mauretanicus mauretanicus* V) (75.8%), AaH II/Lqq

III/Lqh αIT (*L. quinquestriatus hebraeus* αIT) (68.2%) and Lqh II (66.7%). A phylogenetic tree was constructed using a number of representative toxins from each pharmacological group. As shown in Figure 8, BmK αIV was preferentially close to the α-mammal subgroup. An energy-minimized model of BmK αIV was generated as shown in Figure 9. Analysis by PROCHECK program showed that the Ramachandran scores of BmK αIV model are close to those of the AaH II template. The best model showed 94.3% residues in the most favoured regions and 100% in the most favoured or additional allowed regions (Figure 9B). The analysis showed that four clusters of

Figure 8 Unrooted phylogenetic tree of scorpion α -toxins

Twelve aligned sequences from Figure 7 were analysed. The phylogeny was a bootstrap consensus tree based upon the cluster algorithms of the PHYLIP software. The number at the nodes indicates the bootstrap value for 100 replications.

charged amino acids were distributed on the external region of the AaH II and BmK α IV molecular surface (ellipses traced with a broken pink line in Figure 9): region 1, Asp⁸, Asp⁹, Val¹⁰ and His⁶⁴; region 2, Arg¹⁸, Glu²⁴, Glu²⁵ and Lys²⁸; region 3, Lys⁵⁸ and Arg⁶²; region 4, Lys², Asp⁵³, and Arg⁵⁶. However, at the relative position of BmK α IV, the residues were Asp⁸, Asp⁹ and Lys¹⁰ (region 1); Arg¹⁸, Asp²⁴, Asp²⁵ and Lys²⁸ (region 2); Arg⁶² (region 3); Arg² Asp⁵³ and Lys⁵⁴ (region 4) respectively.

DISCUSSION

The modulatory effects of BmK α IV on rNav1.2

The gene encoding a novel neurotoxic polypeptide named BmK α IV was cloned from a genomic DNA library prepared from

BmK venom glands, and the mature toxin was successfully heterologously expressed in *E. coli*. Electrophysiological results showed that both the inactivation and the transient peak amplitude of Na⁺ currents of rNav1.2 expressed in *Xenopus* oocytes could be modulated by BmK α IV (Figure 6), suggesting that rNav1.2 is one of the preferred targets for BmK α IV. Owing to the lack of effect of BmK α IV on the voltage-dependence of activation of rNav1.2 channel (Figure 6B), the binding of BmK α IV to rNav1.2 channels might depend on the conformational changes just before the channel opening induced by depolarization [27].

The receptor site of BmK α IV on sodium channels

The major differences among scorpion neurotoxins came from their toxicity and binding properties [3]. The binding of BmK α IV not only to rat cerebrocortical synaptosomes, but also to cockroach neuronal membranes (Figure 3), was distinct from the binding properties of the other known scorpion α -toxins belonging to α -mammal, α -insect or α -like toxins [3,4]. Moreover, the toxicity of BmK α IV to mammals or insects was much weaker when compared with high selective toxicity of the representative classical α -mammal toxins to mice, α -insect and α -like toxins to cockroach. These results thus suggest that BmK α IV might represent a novel functional subgroup of scorpion α -toxins, even if phylogenetic analysis (Figure 8) showed that BmK α IV was preferentially closer to the α -mammal toxins.

The receptor site of BmK α IV was explored by using a series of sodium-channel-targeted neurotoxins belonging to different groups (Figure 4). The binding of BmK α IV to rat cerebrocortical synaptosomes could be potentially competed for by AaH II, the most active classical α -mammal toxin [28], and veratridine, activators acting on receptor site 2 of sodium channels [29], but not by BmK I, an α -like toxin [30,31], suggesting that the receptor site of BmK α IV on rat brain cerebrocortical sodium channels was similar, but incompletely uniform with site 3 of α -mammal toxin and independent of that of α -like toxin. Moreover, the affinity and dynamic selectivity of BmK α IV to sodium channels were distinct. However, the absent competition of AaH II, veratridine and BmK I on BmK α IV binding to the cockroach neuronal membrane indicated that the receptor site of BmK α IV on insect sodium channels might be similar to, but not identical with, that on rat brain-type sodium channels and also unrelated to that of α -like toxin.

Figure 9 Comparison of the electrostatic potentials of AaH II (A) with BmK α IV (B)

Surface electrostatic potentials are shown from electronegative to electropositive by a red to blue continuous colour range. Two molecules were shown in the same orientation. The charged residues were labelled according to their corresponding position in amino acid sequence. The ellipses traced with a broken pink line show the four clusters of charged amino acids distributed on the external region of the molecular surface.

Despite their lower overall sequence identity, the binding of BmK α IV to rat cerebrocortical and cockroach neuronal membranes could also be competitively inhibited by BmK AS (β -like neurotoxin) [4,32], BmK IT2 (depressant insect-selective neurotoxin) [33,34] and BmK abT (a transitional member between α - and β -type toxins) [35], indicating that the receptor site of BmK α IV might be shared, at least partially, with the putative receptor site 3 of β -like neurotoxins, one of the two non-interacting binding sites of depressant insect neurotoxin and that of transitional neurotoxins. These results thus indicate that the receptor site for BmK α IV on sodium channels is distinct from that of known scorpion neurotoxins.

Structural properties of BmK α IV

The possible structural basis for the distinct binding properties of BmK α IV was pursued. Biochemical experiments and site-directed mutagenesis suggested that the interaction surface of scorpion and sodium channels contained at least two distinct domains [36,37]. Core domain (residues 17, 18, 38 and 44) appeared in α -neurotoxins as well as BmK α IV, which was responsible for recognition of receptor site 3 on sodium channels. NC domain (residues 8–10 and 56–64) was responsible for high affinity and the specificity toward sodium channel subtypes. In this domain, residues Asp⁸-Asp⁹-Lys¹⁰ in BmK α IV were similar to those of AaH II (Asp⁸-Asp⁹-Val¹⁰), whereas it differed in Lqh α IT (Lys⁸-Asn⁹-Tyr¹⁰) and BmK I (Lys⁸-Pro⁹-His¹⁰) in (Figures 7 and 9). Interestingly, the segment (position 56–64) of BmK α IV was similar to that of Lqh α IT, a representative α -insect neurotoxin [3], indicating that this region might be involved in BmK α IV binding to insect sodium channel subtypes. However, residue Phe¹⁷ in Lqh α IT, which might interact directly with cockroach sodium-channel receptor site, was replaced by glycine in most classical α -mammal toxins, as well as in BmK α IV. In addition, the extra sequence Asn⁶⁴-Gly⁶⁵-Gly⁶⁶ of BmK α IV was rarely seen in other known scorpion neurotoxins. Thus the diversities of functional surface formed by the above regions might play important determinants for subtle binding and toxicity profiles of BmK α IV, and indicated that BmK α IV may be a distinct structural and functional hybrid of α -toxins.

Gene organization of BmK α IV and its evolutionary implications

It was found that the intron length of BmK α IV (503 bp) was much longer than that of other known scorpion α -toxins such as AaH I' (α -toxin, 425 bp) and BmK I (α -like toxin, 408 bp) [38,39]. The intron sequence of BmK α IV shared a lower sequence similarity with that of AaH I' (50.5%) and BmK I (49.6%). Also, the content of A + T in BmK α IV (62.0%) was different in AaH I' (74.82%) and BmK I (75%) respectively. Intron size has been found to be associated with protein evolution and expression level in the fruitfly *Drosophila* [40]. It was inferred that the intron might have emerged and evolved separately before the divergence of α - and β -toxins, and the larger intron for BmK α IV may be correlated with its apparent low level of transcription [12,40]. No identical peptide corresponding to the amino acid sequence and molecular mass of BmK α IV was purified from BmK crude venom so far [41]. The much longer intron of BmK α IV provided a possible hint for its lower expression abundance in BmK venom. The longer intron, lower expression and lower toxicity of BmK α IV might reflect the positive selectivity and adaptive evolution process of scorpion BmK species under environmental pressure. In combination with the pharmacological and structural properties, these findings suggest that BmK α IV might be an evolutionary intermediate neurotoxin for α -toxins.

In summary, the analysis of genomic organization, electrophysiological characteristics, binding activity and molecular modelling revealed that BmK α IV represents a novel subgroup or structural and functional hybrid of α -toxins, and might be an evolutionary intermediate neurotoxin in scorpion α -toxin family. Thus BmK α IV is valuable as a new finding in the family of scorpion toxins for studying the adaptive evolution of scorpion species, structure–function divergence and diversity of scorpion neurotoxic polypeptides. Likewise, BmK α IV may be another useful tool to approach physiological and pharmacological dynamic regulation of sodium-channel targets.

This study was supported by the National Basic Research Program of China (2006CB500801), partially by grants from National Nature Sciences Foundation of China (30270428 and 30370446). We thank Professor Herré Rochat and Dr Marie-France Martin-Eauclaire for kindly providing AaH II, and Professor Ai-Hua Liang (Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Biotechnology, Shanxi University, 030006 Taiyuan, China) for her fruitful discussions.

REFERENCES

- Catterall, W. A. (2000) From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* **26**, 13–25.
- Yu, F. H., Westenbroek, R. E., Silos-Santiago, I., McCormick, K. A., Lawson, D., Ge, P., Ferreira, H., Lilly, J., DiStefano, P. S., Catterall, W. A. et al. (2003) Sodium channel β 4, a new disulfide-linked auxiliary subunit with similarity to β 2. *J. Neurosci.* **23**, 7577–7585.
- Gordon, D., Savarin, P., Gurevitz, M. and Zinn-Justin, S. (1998) Functional anatomy of scorpion toxins affecting sodium channels. *J. Toxicol. Toxin Rev.* **17**, 131–159.
- Zuo, X. P. and Ji, Y. H. (2004) Molecular mechanism of scorpion neurotoxins acting on sodium channels: insight into their diverse selectivity. *Mol. Neurobiol.* **30**, 265–278.
- Baloozet, L. (1971) Scorpionism in the Old World. In *Venomous Animals and Their Venoms*, volume III: Venomous Invertebrates (Bucherl, W. and Buckley, E. E., eds.), pp. 349–371, Academic Press, New York.
- Goudet, C., Chi, C.-W. and Tytgat, J. (2002) An overview of toxins and genes from the venom of the Asian scorpion *Buthus martensi* Karsch. *Toxicon* **40**, 1239–1258.
- Bai, Z.-T., Zhao, R., Zhang, X.-Y., Chen, J., Liu, T. and Ji, Y.-H. (2006) The epileptic seizures induced by BmK I, a modulator of sodium channels. *Exp. Neurol.* **197**, 167–176.
- Bai, Z.-T., Zhang, X.-Y. and Ji, Y.-H. (2003) Fos expression in rat spinal cord induced by peripheral injection of BmK I, an α -like scorpion neurotoxin. *Toxicol. Appl. Pharm.* **192**, 78–85.
- Zhang, X.-Y., Bai, Z.-T., Chai, Z.-F., Zhang, J.-W., Liu, Y. and Ji, Y.-H. (2003) Suppressive effects of BmK IT2 on nociceptive behavior and c-Fos expression in spinal cord induced by formalin. *J. Neurosci. Res.* **74**, 167–173.
- Tan, Z.-Y., Xiao, H., Mao, X., Wang, C.-Y., Zhao, Z.-Q. and Ji, Y.-H. (2001) The inhibitory effects of BmK IT2, a scorpion neurotoxin on rat nociceptive flexion reflex and a possible mechanism for modulating voltage-gated Na⁺ channels. *Neuropharmacology* **40**, 352–357.
- Chen, B. and Ji, Y.-H. (2002) Antihyperalgesia effect of BmK AS, a scorpion toxin, in rat by intraplantar injection. *Brain Res.* **952**, 322–326.
- Froy, O. and Gurevitz, M. (2003) New insight on scorpion divergence inferred from comparative analysis of toxin structure, pharmacology and distribution. *Toxicon* **42**, 549–555.
- Goldin, A. L. (2002) Evolution of voltage-gated Na⁺ channels. *J. Exp. Biol.* **205**, 575–584.
- Ji, Y.-H., Mansuelle, P., Terakawa, S., Kopeyan, C., Yanaihara, N., Hsu, K. and Rochat, H. (1996) Two neurotoxins (BmK I and BmK II) from the venom of the scorpion *Buthus martensi* Karsch: purification, amino acid sequences and assessment of specific activity. *Toxicon* **34**, 987–1001.
- Ye, J.-G., Wang, C.-Y., Li, Y.-J., Tan, Z.-Y., Yan, Y.-P., Li, C., Chen, J. and Ji, Y.-H. (2000) Purification, cDNA cloning and function assessment of BmK abT, a unique component from the Old World scorpion species. *FEBS Lett.* **479**, 136–140.
- Ji, Y.-H., Huang, H.-Y., Zhou, C.-W., Liu, Y., Hoshino, M., Mochizuki, T. and Yanaihara, N. (1997) BmK AS, an active scorpion polypeptide, enhances [³H]noradrenaline release from rat hippocampal slices. *Biomed. Res.* **3**, 257–260.
- Ji, Y. H., Hattori, H., Xu, K. and Terakawa, S. (1994) Molecular characteristics of four new depressant insect neurotoxins purified from venom of the scorpion *Buthus martensi* Karsch by HPLC. *Sci. China Ser. B Life Sci. Earth Sci.* **37**, 955–963.

- 18 Ye, J. G., Chen, J., Zuo, X. P. and Ji, Y. H. (2001) Cloning and characterization of cDNA sequences encoding two novel α -like-toxin precursors from the Chinese scorpion *Buthus martensii* Karsch. *Toxicon* **39**, 1191–1194
- 19 Bougis, P. E., Rochat, H. and Smith, L. A. (1989) Precursors of *Androctonus australis* scorpion neurotoxins. Structures of precursors, processing outcomes, and expression of a functional recombinant toxin II. *J. Biol. Chem.* **264**, 19259–19265
- 20 Hassani, O., Loew, D., van Dorsselaer, A., Papandreou, M. J., Sorokine, O., Rochat, H., Sampieri, F. and Mansuelle, P. (1999) Aah VI, a novel, N-glycosylated anti-insect toxin from *Androctonus australis* Hector scorpion venom: isolation, characterisation, and glycan structure determination. *FEBS Lett.* **443**, 175–180
- 21 Reed, L. J. and Muench, H. (1938) A simple method of estimating fifty percent end point. *Am. J. Hyg.* **27**, 493–497
- 22 Dodd, P. R., Hardy, J. A., Oakley, A. E., Edwardson, J. A., Perry, E. K. and Delaunoy, J. P. (1981) A rapid method for preparing synaptosomes: comparison with alternative procedures. *Brain Res.* **226**, 107–118
- 23 Lima, M. E. D., Martin-Eauclaire, M. F., Hue, B., Loret, E., Diniz, C. R. and Rochat, H. (1989) On the binding of two scorpion toxins to the central nervous system of the cockroach *Periplaneta americana*. *Insect Biochem.* **4**, 413–422
- 24 Marti-Renom, M. A., Stuart, A. C., Fiser, A., Sanchez, R., Melo, F. and Sali, A. (2000) Comparative protein structure modeling of genes and genomes. *Annu. Rev. Bioph. Biom.* **29**, 291–325
- 25 Laskowski, R. A., MacArthur, M. W., Moss, D. S. and Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structure. *J. Appl. Crystallogr.* **26**, 283–291
- 26 Nicholls, A., Sharp, K. A. and Honig, B. (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* **11**, 281–296
- 27 Ulbricht, W. (2005) Sodium channel inactivation: molecular determinants and modulation. *Physiol. Rev.* **85**, 1271–1301
- 28 Cestèle, S. and Catterall, W. A. (2000) Molecular mechanisms of neurotoxin action on voltage-activated sodium channels. *Biochimie* **82**, 883–892
- 29 Gordon, D., Martin-Eauclaire, M. F., Cestèle, S., Kopeyan, C., Carlier, E., Khalifa, R. B., Pelhate, M. and Rochat, H. (1996) Scorpion toxins affecting sodium current inactivation bind to distinct homologous receptor sites on rat brain and insect sodium channels. *J. Biol. Chem.* **271**, 8034–8045
- 30 Li, Y.-J. and Ji, Y.-H. (2000) Binding characteristics of BmK I, an α -like scorpion neurotoxic polypeptide, on cockroach nerve cord synaptosomes. *J. Peptide Res.* **56**, 195–200
- 31 Chen, J., Tan, Z.-Y., Zhao, R., Feng, X.-H., Shi, J. and Ji, Y.-H. (2005) The modulation effects of BmK I, an α -like scorpion neurotoxin, on voltage-gated Na^+ currents in rat dorsal root ganglion neurons. *Neurosci. Lett.* **390**, 66–71
- 32 Li, Y.-J., Liu, Y. and Ji, Y.-H. (2000) BmK AS: new scorpion neurotoxin binds to distinct receptor sites of mammal and insect voltage-gated sodium channels. *J. Neurosci. Res.* **61**, 541–548
- 33 Li, Y.-J., Tan, Z.-Y. and Ji, Y.-H. (2000) The binding of BmK IT2, a depressant insect-selective scorpion toxin on mammal and insect sodium channels. *Neurosci. Res.* **38**, 257–264
- 34 Chai, Z.-F., Bai, Z.-T., Liu, T., Pang, X.-Y. and Ji, Y.-H. (2006) The binding of BmK IT2 on mammal and insect sodium channels by surface plasmon resonance assay. *Pharmacol. Res.* **54**, 85–90
- 35 Ji, Y.-H., Wang, W.-X., Wang, Q. and Huang, Y.-P. (2002) The binding of BmK abT, a unique neurotoxin, to mammal brain and insect Na^+ channels using biosensor. *Eur. J. Pharmacol.* **454**, 25–30
- 36 Zilberberg, N., Froy, O., Loret, E., Cestèle, S., Arad, D., Gordon, D. and Gurevitz, M. (1997) Identification of structural elements of a scorpion α -neurotoxin important for receptor site recognition. *J. Biol. Chem.* **272**, 14810–14816
- 37 Karbat, I., Frolow, F., Froy, O., Gilles, N., Cohen, L., Turkov, M., Gordon, D. and Gurevitz, M. (2004) Molecular basis of the high insecticidal potency of scorpion α -toxins. *J. Biol. Chem.* **279**, 31679–31686
- 38 Delabre, M. L., Pasero, P., Marilley, M. and Bougis, P. E. (1995) Promoter structure and intron–exon organization of a scorpion α -toxin gene. *Biochemistry* **34**, 6729–6736
- 39 Xiong, Y. M., Ling, M. H., Wang, D. C. and Chi, C. W. (1997) The cDNA and genomic DNA sequences of a mammalian neurotoxin from the scorpion *Buthus martensii* Karsch. *Toxicon* **35**, 1025–1031
- 40 Marais, G., Nouvellet, P., Keightley, P. D. and Charlesworth, B. (2005) Intron size and exon evolution in *Drosophila*. *Genetics* **170**, 481–485
- 41 Wu, H., Wu, G., Huang, X., He, F. and Jiang, S. (1999) Purification, characterization and structural study of the neuro-peptides from scorpion *Buthus Martensi* Karsch. *Pure Appl. Chem.* **71**, 1157–1162

Received 5 January 2006/16 June 2006; accepted 27 June 2006

Published as BJ Immediate Publication 27 June 2006, doi:10.1042/BJ20060035