BmBKTx1, a Novel Ca\textsuperscript{2+}-activated K\textsuperscript{+} Channel Blocker Purified from the Asian Scorpion Buthus martensi Karsch*

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BmBKTx1 is a novel short chain toxin purified from the venom of the Asian scorpion Buthus martensi Karsch. It is composed of 31 residues and is structurally related to SK toxins. However, when tested on the cloned rat SK2 channel, it only partially inhibited rSK2 currents, even at a concentration of 1 μM. To screen for other possible targets, BmBKTx1 was then tested on isolated metameric dorsal unpaired median neurons of Locusta migratoria, in which a wide variety of ion channels are expressed. The results suggested that BmBKTx1 could specifically block voltage-gated Ca\textsuperscript{2+}-activated K\textsuperscript{+} currents (BK-type). This was confirmed by testing the BmBKTx1 effect on the α subunits of BK channels of the cockroach (pSlo), fruit fly (dSlo), and human (hSlo), heterologously expressed in HEK293 cells. The IC\textsubscript{50} for channel blocking by BmBKTx1 was 82 nM for pSlo and 194 nM for dSlo. Interestingly, BmBKTx1 hardly affected hSlo currents, even at concentrations as high as 10 μM, suggesting that the toxin might be insect specific. In contrast to most other scorpion BK blockers that also act on the Kv1.3 channel, BmBKTx1 did not affect this channel as well as other Kv channels. These results show that BmBKTx1 is a novel kind of blocker of BK-type Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels. As the first reported toxin active on the Drosophila Slo channel dSlo, it will greatly facilitate studying the physiological role of BK channels in this model organism.

Most scorpion toxins specific for K\textsuperscript{+} channels (KTx) are short chain peptides comprising 23–47 amino acid residues cross-linked by 3 to 4 disulfide bridges. All these toxins are composed of an α-helix connected to a double- or triple-stranded β-sheet by highly conserved disulfide bridges (1). The scorpion KTx have been classified into three subfamilies: α-, β-, and γ-KTx (2, 3). Among them, the α-KTx have become valuable tools for testing pharmacological, physiological, biochemical, biophysical, and even structural characteristics of K\textsuperscript{+} channels and their associated ionic currents (1). The classification of KTx is mainly based on their primary structures rather than on their pharmacological profiles, because the pharmacology of some toxins still remains to be determined and only a few of the characterized toxins are selective for only one K\textsuperscript{+} channel subtype (2). Thus, highly selective toxins deserve particular attention as tools in research and potential resources for drug development.

Big conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} currents, also termed BK or Slo currents, are activated both by an increase in cytosolic Ca\textsuperscript{2+} concentration and by depolarization (4). BK channels were first studied in smooth muscle cells, where they are particularly abundant and play a key role in setting the contractile tone. Many nerve cells also contain Ca\textsuperscript{2+}-activated K\textsuperscript{+} currents that play a key role in controlling excitability and action potential waveform. These currents prevent excessive Ca\textsuperscript{2+} entry, and are involved in the inhibition of neurotransmitter release (5). Furthermore, BK channels are essential for innate immunity (6). To date, no human disease has been found to be firmly associated with BK channels (7), but their ubiquitous presence in excitable and non-excitable cells underlies their fundamental role in coupling chemical signaling with electrical signaling.

BK channels are widespread in the animal kingdom. Pore-forming α subunits of BK channels (Slo) have been cloned from the nematode Caenorhabditis elegans, insects (Drosophila melanogaster, and Periplaneta americana), and mammals. For mammalian BK channels, the α-subunit may be linked to a regulatory β-subunit that affects channel kinetics and pharmacology (8).

Some scorpion α-KTx, such as charybdotoxin (ChTx) and iiberotoxin (IbTx), are frequently used as tools to block BK channels. They consist of more than 36 amino acid residues connected by three disulfide bridges, and show high sequence homology. Nevertheless, only IbTx and limbatoxin were found to block BK channels selectively (9). ChTx and others affect both BK channels and the voltage-gated Kv1.3 channel (10). The pharmacophores of ChTx responsible for the interactions with BK and Kv channels seem to be overlapped in the C-terminal β-sheet region, suggesting that the outer vestibules.
of BK and Kv channels may, at least partially, share the same topology (11, 12). Obviously, research on BK channels would benefit from identifying more highly selective BK blockers. Moreover, there is a lack of a specific blocker for dSlo, the Drosophila BK channel, because neither ChTx nor IbTx affect this channel (13).

The Asian scorpion BmK has been used for many years in traditional Chinese medicine. Its venom has recently been investigated in detail, and more than 70 different neurotoxins including 18 KTx have been isolated from it (14). Here, we present the purification and characterization of a novel BK blocker, designated BmBKTx1, from the Asian scorpion Buthus martensi Karsch (BmK). We have tested BmBKTx1 on various voltage-gated and Ca2+-activated K+ currents and observed a potent blocking effect on insect BK currents.

**EXPERIMENTAL PROCEDURES**

**Toxin Purification and Sequencing**

The venom of the Asian scorpion BmK was first fractionated on a Sephadex G-50 column as previously described (15). Peak 2, containing various neurotoxins, was applied to a DEAE Sephacel column (3 × 5 cm) previously equilibrated with 20 mM NaCl, 0.1 M NaHCO3 buffer; pH 10.5. The breakthrough fraction was pooled and loaded onto a semi-preparative reverse-phase HPLC C18 column (1 × 25 cm, Beckman) equilibrated with buffer A (0.1% trifluoroacetic acid in water) at a flow rate of 2 ml/min. Proteins were eluted by a two-step gradient system: 0 to 36% buffer B (70% acetonitrile in buffer A) for 36 min, and 37 to 46% buffer B for 15 min. Fraction 3 from the C18 column was applied to a Mono-S column (0.5 × 5 cm, Amersham Biosciences) equilibrated with 50 mM HAc-NaAc buffer, pH 4.3, at a flow rate of 1 ml/min. Elution was carried out with a gradient of 0–0.6 M NaCl in the acetate buffer for 30 min. The component isolated in the third peak (BmBKTx1) was used in the subsequent studies. The complete amino acid sequence of BmBKTx1 was obtained from a signal sequencing run on an Applied Biosystems 491 pulsed liquid-phase sequencer.

**Chemical Synthesis of BmBKTx1**

BmBKTx1 was synthesized on Boc-Lys (2Cl2)OCH2-phényl-acetamidomethyl resin using a custom-modified, machine-assisted chemistry tailored from the published in situ DIEA neutralization/O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate activation protocol for Boc solid-phase peptide synthesis (16). The side chain protection sides were used: Cys(4MeBzl), Asp(OcHxI), Lys(2Cl2), Asn(xanthy1), Arg(tosyl), Ser(Bzl), and Tyr(Brz). After chain assembly, the peptide was cleaved and deprotected by HF for 12 h in the presence of 5% p-cresol/thiocresol (1:1) at 0 °C, followed by precipitation with cold ether. The crude product was purified by reversed-phase HPLC to homogeneity, and its molecular weight ascertained by electrospray ionization mass spectrometry.

Oxidative folding of the purified BmBKTx1 was performed by dissolving the peptide at 3 mg/ml in 6 M guanidine HCl containing 15 mM reduced glutathione and 1.8 mM oxidized glutathione, followed by a rapid 6-fold dilution with 0.25 M NaHCO3. The folding/disulfide formation proceeded quantitatively at room temperature overnight, and the final product was purified by HPLC and lyophilized.

**Patch Clamp Recording on Locust Dorsal Unpaired Median (DUM) Neurons**

Cell Preparation—Adult migratory locusts (Locusta migratoria) of both sexes were taken from the crowded laboratory colony 2 to 10 days after imaginal moult. Animals were reared at ~32 °C on a 14:10 h light/dark cycle on a diet of grass and oatmeal (17).

Isolated DUM neuronal cell bodies from the metathoracic ganglion were prepared as described previously (18). Briefly, the dorsal median region from ganglia of 6 to 7 animals was removed and subjected to collagenase/dispase (2 mg/ml) treatment. The cells were centrifuged and subsequently washed three times. Cells were isolated by repetitive suction through a pipette tip, plated on Nunc Petri dishes, and incubated overnight at 28 °C under 5% CO2. All products were obtained from Invitrogen except the collagenase/dispase mixture (Roche Applied Science). Patch Clamp Recording—K+ currents in isolated DUM neurons of L. migratoria at room temperature were studied using the whole cell patch clamp technique (19). The electrodes were filled with standard intracellular solution (SIS) containing concentrations of (in mM) 160 K+-glucuronate, 6.5 NaCl, 1 CaCl2, 2 MgATP, 50 glucose, 10 EGTA, and 10 HEPES, at pH 6.65. The standard extracellular solution (SSE) contained concentrations of 172.5 mM NaCl, 6.5 KCl, 2 CaCl2, 7.7 MgCl2, 13 glucose and 10 HEPES, at pH 6.80. To separate K+ currents, tetrodotoxin (TTX) was added to the extracellular solution (standard extracellular solution + TTX), to a concentration of 100 nM, to block the voltage-gated Na+ current in the DUM neurons. When necessary, Ca2+ currents were blocked by further addition of Ca2+-free to the extracellular solution (K+ES = standard extracellular solution + TTX + Ca2+-free) to a concentration of 1 mM. K+IS was equivalent to standard intracellular solution except containing an increased free Ca2+ concentration of 5 µM (using 2 mM EGTA and 1.48 mM CaCl2) to potentiate the Ca2+-activated K+ currents in DUM neurons. The free Ca2+ concentration was calculated using the program CaBuf, taking into account the concentrations of Ca2+, Mg2+, EGTA, and ATP. 400 nM IbTx was used to block BK-type Ca2+-activated K+ currents. Application and washout of different extracellular solutions was accomplished with a bath perfusion system.

Voltage clamp experiments and data acquisition were performed with a PC-controlled EPC 9 or EPC 10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany), as previously described (18). The cells were clamped at a holding potential of ~100 mV. Capacitative and leak currents were compensated using an online routine provided by the PULSE software (HEKA Elektronik, Lambrecht, Germany), the residual currents being eliminated through the P/4 protocol. The outward currents recorded in the DUM neurons showed some variability in magnitude, probably because of differences in the sizes (ranging from 40 to 60 µm in diameter) of the DUM neurons used in the experiments.

**Patch Clamp Recording on SK and Slo Channels Expressed in HEK293 Cells**

Transfection and Expression—For electrophysiological recording, SK and Slo channels were transiently expressed in HEK293 cells. Cells were cultured at a density of ~2 × 10⁵/35-mm dish, and transfected with 1 µg of channel DNA as specified below, and 0.5 µg of pEGFP-C1 (Clontech) using the SuperFect reagent (Qiagen, Hilden, Germany). We expressed the Paraplaneta Slo channel (pSlo) using pSlo/pDNA3.1 (splicing form A1/C2/E1/G3/I0), the human Slo channel (hSlo) using pSlo/pcDNA3.1, and the rat SK2 channel (rSK2) using rSK2/pDNA3.1.

**Patch Clamp Recording on HEK293 Cells**—Whole-cell currents from HEK293 cells expressing Slo or SK channels were measured at room temperature using borosilicate pipettes with resistances of 2 to 5 MΩ. Current measurements and data acquisition were performed with an EPC 9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany), which was controlled by PULSE software (HEKA Elektronik). Currents were sampled at 10 kHz and filtered at 2.9 kHz. Capacitative and leakage currents were compensated using a P/4 protocol (the holding potential for leakage measurement was ~110 mV). Series resistance error was compensated by ~75%. The bath solution for measuring SK currents contained concentrations of (in mM) 150 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 0.33 NaH2PO4, 2 sodium pyruvate, 10 glucose, and 10 HEPES. For measuring SK currents, the bath solution differed from the above by containing 110 mM NaCl and 30 mM KC1. The pipette solution contained concentrations of 140 mM KC1, 4 NaCl, 2 MgATP, and 10 HEPES; the free Ca2+ concentration was measured with a calcium-sensitive electrode (KWIK tips; WPI, Berlin, Germany), and adjusted to 2.5 µM for measuring SK and hSlo currents, and to 25 µM for measuring insect Slo currents. The pH of the bath solution was adjusted to 7.4, and that of pipette solution to 7.25. Liquid-junction potentials between pipette and bath solution were taken into account before closing the seal. The holding potentials (VHOLD) for Slo and SK current measurements were ~90 and ~40 mV, the K+ equilibrium potentials amounting to ~84 and ~39 mV, respectively. For toxin application, the bath perfusion system BPS4 (ALa, Westbury, NY) was used.

**Voltage Clamp Recording on Various Cloned K+ Channels Expressed in Xenopus laevis Oocytes**

**Injection and Expression—Stage V–VI. laevis oocytes were isolated as previously described (20). The oocytes were defolliculated by treatment with 2 mg/ml collagenase in zero-calcium ND-96 solution. Between 2 and 24 h after defolliculation, oocytes were injected with 50 nL of 1–100 ng/ml cRNA using a Drummond micrometer. The oocytes were then incubated in ND-96 solution at 18 °C for 1–4 days. ND-96 solution, pH 7.5, contained concentrations of (in mM) 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES, supplemented with 50 mg/liter gentamycin sulfate (only for incubation). The in vitro synthesis of
cRNAs encoding Kv1.1, Kv1.2, Kv1.3, and Kir2.1, respectively, were performed as previously described (20, 21).

To prepare the cRNAs of HERG, KCNQ1, and minK, plasmids containing these channel genes were first linearized by restriction endonucleases. The pSP64 plasmids containing the HERG gene were linearized with EcoRI. The pEXO plasmids containing the mKCNQ1 clone were linearized with BamHI. The original pBlueScript SK+/H11002 vector containing the mIsK gene was first subcloned into the pGEM HE vector. Then the pGEM HE vector was linearized with PstI. Using the linearized plasmids as templates, cRNAs were synthesized in vitro using the large scale T7 or SP6 mMESSAGE mMACHINE transcription kit.

Voltage Clamp Experiments—Whole cell currents from oocytes were recorded using the two-microelectrode voltage clamp technique. Voltage and current electrodes (0.4–2 MΩ) were filled with 3 M KCl. The bath solution was ND-96, pH 7.5. Current records were sampled at 0.5-ms intervals after low pass filtering at 1 kHz. Linear components of capacity and leak currents were not subtracted. All experiments were performed at room temperature (19–23°C).

Data Analysis

Results are mostly shown as mean ± S.E., n being the number of experiments. The significance of differences between two means was calculated with the Wilcoxon matched pairs test using Prism software (Graphpad Software, San Diego, CA). Nonlinear fitting procedures of data sets were performed using Origin 6.0 (Micoral Software, Northampton, MA) or IGOR (WaveMetrics, Lake Oswego, OR) software. The significance of the differences between parameters obtained by curve fitting (mean ± S.E.) was calculated using the Student’s t test with Welch correction (Prism, Graphpad Software). Differences in the mean values were considered significant at probability <0.05.

Concentration-response relationships were fitted according to: $I_{\text{norm}} = 1/I_0 + \frac{\text{[BmBKTx1]}/EC_{50}}{1 + \text{[BmBKTx1]}/EC_{50}}$, where $I_{\text{norm}}$ is the normalized peak current (see below) and [BmBKTx1] is the concentration of BmBKTx1. The parameters to be fitted were concentration of half-maximal effect ($EC_{50}$), the Hill coefficient ($p$). For calculation of $I_{\text{norm}}$, peak currents measured in the presence of the toxin (after equilibration of toxin effect) were normalized to peak currents obtained under control conditions.

RESULTS

Toxin Purification, Protein Sequencing, and Chemical Synthesis—As described under “Experimental Procedures,” a novel toxin was purified from the crude venom of the Asian scorpion BmK by gel filtration chromatography, anion-exchange chromatography, reverse-phase HPLC, and cation-exchange HPLC (Fig. 1). The purified toxin was sequenced by Edman degradation on an Applied Biosystem 491 pulsed liquid sequencer. The results indicate that the toxin is a short chain peptide composed of 31 amino acid residues cross-linked by three disulfide bridges. Given its potent activity on the BK channel (see following sections), this toxin was designated as BmBKTx1. Because the native BmBKTx1 is scarce in the crude venom (less than 1%), the toxin was synthesized to obtain enough material for further characterization. The native and synthetic BmBKTx1 were co-eluted on reverse-phase HPLC, indicating their identity.

In terms of peptide length, BmBKTx1 is similar to scorpion SK blockers such as scyllatoxin (LeTxI), which is also composed of 31 residues (22, 23), but shorter than the classical Kv/BK blockers, composed of more than 36 residues (24, 25). However, BmBKTx1 lacks a pronounced sequence homology with SK blockers. On the other hand, its C-terminal region is similar to that of α-KTx1.x toxins active on Kv/BK (Fig. 2). The amino acid sequence of the toxin had already been deposited in the
NCBI Protein Data base before it was classified and functionally
described (Protein Data Bank code P33407). Named
BmK37 at that time, it was also included in a phylogenetic
tree in a recent review on the interaction between K+ channels and
scorpion toxins (1), where it was assigned to a cluster of one
group of the Kv1.x channel blockers that are closely related to
the SK channel blockers.

Effect of BmBKTx1 on SK2 Channels—Considering the similarity
of the amino acid number of BmBKTx1 and SK channel
toxins, we tested its effect on rat SK2 channels (rSK2) heter-
ologously expressed in HEK293 cells. Whereas specific SK tox-
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group of the Kv1.x channel blockers that are closely related to
the SK channel blockers.
FIG. 3. The effect of BmBKTx1 on rSK2 channels heterologously expressed in HEK293 cells. Application of 1 μM BmBKTx1 caused incomplete block of the rSK2 channel current. Currents were activated by 2.5 μM free [Ca^{2+}] in the pipette solution and recorded with voltage ramps of 400 ms from −100 to +100 mV. For analysis, currents obtained at +100 mV were used. A, registration samples of rSK2 currents obtained on voltage ramps as indicated. The holding potential of −40 mV corresponds to the equilibrium potential for K⁺. The current registered 2 min after establishing the whole cell configuration (Control) was reduced to the considerably lower level by 1 μM BmBKTx1 within 8 min of incubation. The residual current, resistant to 1 μM BmBKTx1, was blocked by 50 nM apamin within 3 min of incubation. B, development of current block by BmBKTx1 in a HEK293 cell. The monoexponential curve well fitted to the data is described by a time constant of 200 s. Application of 50 nM apamin after saturation of the BmBKTx1 effect caused an additional current reduction. Bars indicate the presence of toxins. C, fraction of total current blocked by 1 μM BmBKTx1 and by further administration of 50 nM apamin. The BmBKTx1-sensitive current fraction was measured 8 min after toxin application. The fraction of current blocked by apamin in the continuous presence of BmBKTx1 was measured 3 min after apamin application. Columns, means of 8 cells; bars, S.D. On average, 1 μM BmBKTx blocked −64% of the total rSK2 current.

FIG. 4. The effect of BmBKTx1 on the ionic currents of isolated locust DUM neurons. A–C, the currents were recorded during a 40-ms depolarizing voltage step to +40 mV, from a holding potential of −100 mV (x axis, 5 ms; y axis, 10 nA). A, the current traces recorded in standard intracellular solution and standard extracellular solution are shown. A.2, when 100 nM TTX is added to the extracellular medium, a similar effect is produced. B, currents were recorded in standard intracellular solution and KCdES. Cd²⁺ was added to block Ca²⁺ currents directly, and to block Ca²⁺-activated K⁺ currents indirectly. C, Ca²⁺ currents were blocked by Cd²⁺ (KCdES), and the intracellular Ca²⁺ concentration was raised to 5 μM (KCaIS). D, for all three experimental conditions (the numbers of the corresponding panels are given), the currents in the presence of the toxin were normalized to the control current, and means for 6 to 11 cells were taken. Significant differences between control and test values are indicated by the asterisk (*), and between two test values by the § (p < 0.05, for one sample t test and Welch corrected unpaired t test, respectively). E, pharmacological separation of the Ca²⁺-activated outward currents using 1 mM Cd²⁺ to block influx of Ca²⁺ in locust DUM neurons. All currents were recorded in the presence of TTX. E.1, by subtracting the current after addition of 1 mM Cd²⁺ (E) from the recordings in control conditions (D), the Ca²⁺-activated outward currents were obtained. E.2, a family of Ca²⁺-activated K⁺ currents obtained using the same method.
Slo currents (Fig. 5A), even at concentrations as high as 10 μM (Fig. 5E). However, the insect Slo channels from Drosophila (dSlo) and Periplaneta (pSlo) did exhibit sensitivity to BmBKTx1 (Fig. 5B, C, and E). Compared with that of rSK2, the block for these insect channels developed more quickly. The time constants of the current decay elicited by 1 μM BmBKTx1 were 105 s for dSlo and 65 s for pSlo (Fig. 5D). The block was partially reversible upon washout. The concentration dependence of the toxin effect is described by IC50 values of 194 and 82 nM and Hill coefficients of 0.56 and 0.5 for dSlo and pSlo, respectively (Fig. 5E). These Hill coefficients are comparable with that of IbTx for pSlo, Hill coefficient 0.65 (29), and suggest possible negative cooperativity of the binding of BmBKTx1 to BK channels.

In contrast with pSlo (29) and mammalian Slo channels, dSlo is resistant to the prototypic BK channel blockers ChTx and IbTx (13). BmBKTx1 is thus the first known peptide toxin blocking the dSlo channel. Moreover, the resistance of hSlo to BmBKTx1 seems to indicate an insect specificity for this toxin.

**Selectivity Pattern**—To study the selectivity of BmBKTx1, it was tested on various other cloned K+ channels. Interestingly, BmBKTx1 had no effect on the voltage-gated K+ channels Kv1.1, Kv1.2, Kv1.3, HERG, and K_LQT1, or on the inward rectifier K+ channel Kir2.1 (all expressed in *X. laevis* oocytes). So far, only IbTx and limbatotoxin have been found to be selective for BK channels, whereas other scorpion BK blockers also blocked Kv1.3 channels (9, 10). As shown in Fig. 5F, 1 μM BmBKTx1 had almost no effect on Kv1.3, whereas 0.1 μM BmBKTx1 blocked more than 50% of the pSlo current. Thus, BmBKTx1 is the third scorpion toxin selectively acting on BK channels but not on Kv channels.

**DISCUSSION**

K+ channels play a key role in cellular excitability and signal transduction. Exploring their structure and function has led to the design of many therapeutic compounds (7). High affinity blockers isolated from scorpion venom have been used to elucidate pharmacological and structural characteristics of K+ channels. To obtain yet more useful tools for K+ channel studies, it is important to continue the search for new, specific toxins. In the present study we investigated effects of a new scorpion toxin on various channels. Our experiments on Kv and KCa channels revealed that BmBKTx1 selectively acts on Ca2+-activated K+ channels. Interestingly, BmBKTx1 blocked both SK and BK channels. Although its effect on the SK channel was less pronounced than on insect BK channels, BmBKTx1 is the first toxin acting on both of these different KCa channel types. It might be speculated that BmBKTx may also affect IK channels. If this were to be true, this toxin could be useful in screening for KCa channels in a variety of cells. Further investigations will be needed to address this point.

**Mammalian/Insect Specificity**

Although the Slo gene encoding big conductance Ca2+-activated K+ channels is highly conserved throughout evolution, mammalian and insect Slo channels nevertheless exhibit small differences in their pore regions (Fig. 6). Previous studies have shown that the critical determinants for ChTx/IbTx sensitivity are located close to the pore region of Slo channels (9, 30, 31), and even a single point mutation in this region could dramatically influence the scorpion toxin affinity (29, 32). Table I summarizes the ability of ChTx and BmBKTx1 to block different mammalian and insect Slo channels. The IC50 values of ChTx for mammalian Slo channels (36 and 7.4 nM for hSlo and mSlo, respectively) are clearly lower than those for insect Slo channels (150 nM and >5 μM for pSlo and dSlo, respectively) (29, 32). Interestingly, changing one residue, in position 290 of dSlo, from Thr to Glu, the equivalent residue in mammalian Slo, greatly increased the channel sensitivity to ChTx (IC50 23 nM) (32). Moreover, when position 285 in pSlo was changed to Lys, present at the equivalent position in mammalian Slo, ChTx was more effective on it than on wild type pSlo (29). Therefore, ChTx seems to have higher mammalian specificity.
and short chain toxins, despite their different targets. Specificity would seem to be a common feature in both long chain studied and described in detail (33). Therefore, species specificity in BmBKTx1, the two basic residues, Arg9 and 37). Although no basic residues could be found at the equivalent position in BmBKTx1, the two basic residues, Arg9 and Lys11, located at the N-terminal -helical face (1). Hence, BmBKTx1 might also have two separate functional faces corresponding to its BK and SK activities.

In contrast, BmBKTx1 blocked insect Slo channels at nanomolar concentrations, but showed no effect on hSlo, implying that this toxin might have an insect specificity, at least for BK channels. Given the sequence identity in the pore region of hSlo with those of rSlo and mSlo (Fig. 6), one might expect no effect of BmBKTx1 on rSlo and mSlo. Further experiments on other mammalian Slo channels are needed to test the hypothesis of insect specificity, and site-directed mutagenesis will clarify the structural basis for species specificity.

To our knowledge, this is the first detailed report on mammalian/insect specificity of scorpion short chain K⁺ channel toxins. The issue has, in general, been poorly addressed in previous studies. However, at least the species selectivity of scorpion long chain toxin active on Na⁺ has been well studied and described in detail (33). Therefore, species specificity would seem to be a common feature in both long chain and short chain toxins, despite their different targets.

**Structure-Function Relationship**

The First Scorpion Toxin Active Both on BK and SK—It is already known that BK toxins block the pore region of the channel with their C-terminal -sheet face, whereas SK toxins block the intermediate region of the channel with their N-terminal -helix face (1). Hence, BmBKTx1 might also have two separate functional faces corresponding to its BK and SK activities.

Compared with other scorpion BK blockers, BmBKTx1, composed of only 31 amino acid residues, is the shortest one. All others BK blockers are composed of more than 36 amino acid residues. Despite their sequence diversity in the N-terminal region, their C-terminal regions show a very high homology (Fig. 2). The solution structure of BmBKTx1 has recently been determined by NMR (34). Superimposition of the three-dimensional structure of BmBKTx1 with that of ChTx (35) demonstrates that, except for the N-terminal region, the backbones of the two toxins are closely matched (Fig. 7). However, there are small differences in the -sheet faces of BmBKTx1 and ChTx, which might well explain their different activities on Slo channels.

Compared with other SK blockers, BmBKTx1 has the same length but low sequence homology. Previous studies indicate that three basic residues, located at the -helical face at positions 6, 7, and 13, are crucial for SK toxin binding (22, 26, 36, 37). Although no basic residues could be found at the equivalent positions in BmBKTx1, the two basic residues, Arg⁵ and Lys⁸¹, located at the N-terminal -helical face, might play the same role as the basic residues in LeTx1 and its analogues.

The Absence of Kv1.3 Blocking Activity—As stated above, unlike the other scorpion blockers of BK channels such as ChTx, BmBKTx1 does not block the Kv1.3 channel. The only other exceptions are the BK-specific IbTx and limitoxatin. Site-directed mutagenesis and the toxin-channel docking model have revealed that Gly³⁰ in IbTx plays a key role in its pharmacological selectivity (9, 31). The mutation G30N induces blocking of Kv1.3. In ChTx, the equivalent position is occupied by the large residue Asn (Asn³⁰), which was found to interact tightly with Asp³⁸³ in Kv1.3. The Asn³⁰-Asp³⁸³ pair is one of the key interaction pairs for toxin binding. The mutation N30G in BmBKTx1 caused a 840-fold decrease in its affinity for Kv1.3 (11). The corresponding position in BmBKTx1 is Asn³². Asn at this position should enhance its binding to Kv1.3. Nevertheless, BmBKTx1 does not block this channel, i.e. there seem to be residues in other positions that prevent BmBKTx1 from binding to the Kv1.3 channel. BmBKTx1 and ChTx share the same cysteine-stabilized / scaffold, and their backbones match very well, except for their N termini (Fig. 7). Thus, the interaction surfaces of ChTx to Kv1.3 might be used to predict and to explain the interaction (or its absence) between BmBKTx1 and Kv1.3. Table II lists the interaction pairs for ChTx and the Kv1.3 channels obtained experimentally, and the equivalent residues in BmBKTx1.

| ChTx pairs between ChTx and Kv1.3 and equivalent residues in BmBKTx1 |
|-----------------|-----------------|-----------------|
| Thr⁴/Thr⁴ | Tyr⁵/Ser⁵ | Gly³⁷⁵ |
| Arg⁵⁰ | Asp³⁸³ | Asp³⁸³ |

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instance, mutation F425G takes away the bulky aromatic residue in the interaction pair, and as a consequence, ChTx binding affinity is enhanced 2000 times (11).

Arg \(_2\) in ChTx interacts with Asp\(^{381}\) in Kv1.3 via electrostatic interaction. The mutations R25D in ChTx and D381K in the channel decrease the toxin binding affinity 1400- (11) and -500-fold (39), respectively. In BmBKTx1, Arg\(^{25}\) corresponds to Ser\(^{18}\), which is an uncharged residue that cannot form a salt bridge with Asp\(^{381}\) (Fig. 7C). Furthermore, this residue is not well solvent-exposed. Thus, an important pair is not functional in the BmBKTx1-Kv1.3 interaction.

Considering that the unfavorable contacts discussed above can have effects that, on their own, are large enough to prevent BmBKTx1 from binding to Kv1.3, one can understand why BmBKTx1 does not block Kv1.3 even if the pair Asn\(^{22}\) in BmBKTx1 from binding to Kv1.3, one can understand why BmBKTx1 is novel specific tool to study BK channels in Drosophila and other insect organisms, and to enhance our understanding of scorpion toxins.

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