BmP09, a ‘Long-Chain’ Scorpion Peptide Blocker of BK Channels

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Running title: A new scorpion toxin selectively against BK channels

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A novel “long-chain” toxin BmP09 has been purified and characterized from the venom of Chinese scorpion *Buthus martensi* Karsch. The toxin BmP09 is composed of 66 amino acid residues, including eight cysteines, with a M.W. 7721.0 Da. Compared with the BmK AS-1 as a Na\(^+\) channel blocker (M.W. 7704.8 Da), the BmP09 has an exclusive difference in sequence by an oxidative modification at the C-terminus. The sulfoxide Met66 at the C-terminus brought the peptide a dramatic switch from a Na\(^+\) channel blocker to a K\(^+\) channel blocker. Upon probing the targets of the toxin BmP09 on the isolated mouse adrenal medulla chromaffin cells (MACCs), where a variety of ion channels coexists, we found that the toxin BmP09 specifically blocked large conductance Ca\(^{2+}\)- and voltage-dependent K\(^+\) channels (BK), but not Na\(^+\) channels at a range of 100 nM concentration. This was further confirmed by blocking directly the BK channels encoded with mSlo1 α-subunits in Xenopus oocytes. The half-maximum concentration EC\(_{50}\) of BmP09 was 27 nM and the Hill coefficient was 1.8. In outside-out patches, the 100 nM BmP09 reduced ~70% currents of BK channels without affecting the single-channel conductance. In comparison with the “short-chain” scorpion-peptide toxins such as Charybdotoxin (ChTX), the toxin BmP09 behaves much better in specificity and reversibility, and thus it will be a more efficient tool for studying BK channels. A 3D simulation between a BmP09 toxin and a mSlo channel shows that the Lys41 in BmP09 lies at the center of the interface and plugs into the entrance of the channel pore. The stable binding between the toxin BmP09 and the BK channel is favored by aromatic π-π interactions around the center.
INTRODUCTION

Large conductance Ca\(^{2+}\)- and voltage-dependent potassium (MaxiK, BK) channels are thought to play a primary role in a link between the membrane potential and the cellular calcium homeostasis. BK channels are very abundant in many tissues from pancreas to smooth muscle to brain (1). Natural toxins are among the most potent and important tools for studying the functions and structure of ion channels. Various species such as sea anemone, snakes, cone snails, spiders and scorpions, possess of ion-channel toxins in their venoms (2). \(\kappa\)-BtX coming from the venom of a worm-hunting cone snail enhances the currents of BK (3). Another toxin from medicine herb DSH-I also increases the BK currents when \(\alpha\) subunit coexists with its \(\beta\) subunit (4). Scorpion venoms are rich source of fascinating neurotoxins, which bond with high affinity and specificity to various ion channels and thus widely serve as useful tools in probing the protein mapping of ion channels and clarifying the molecular mechanism involved in the signal transmission and channel gating. Some of peptidyl scorpion toxins such as Charybdotoxin (ChTX), Iberiotoxin (IbTx) and Slotoxin (SloTx) also block the BK currents encoded by both the Slo1 \(\alpha\) subunits and the \(\beta\) subunits but with a higher EC\(_{50}\) (5, 6). Those toxins, in common, are very poor reversibility, which give us a really hard time to study functions of BK currents, especially in current-clamp experiments, even though this property is often used to identify the existence of \(\beta\) subunits (7-9).

Recently, Xu et al. (10) finds another scorpion toxin BmBKTx1 which blocks pSlo (82 nM) and dSlo (194 nM), but not for hSlo. According to its specific characteristics, BmBKTx1 can be used to identify different subunits involving in BK channels. So far more than 120 peptide modulators of ion channels have been isolated from scorpion venoms. Most of the scorpion toxins blocking K\(^+\) channels (KTx) are short peptides (22-43 amino acids residues) with a well-conserved
three-dimensional structure stabilized by three or four disulfide bridges (11).

The Chinese scorpion Buthus martensi Karsch (BmK) has been used as traditional medicine in China for more than one thousand years, especially for treatments in neural diseases, such as apoplexy, hemiplegia, and facial paralysis (12). Indeed, over the past decade, more than 70 different peptides, toxins or homologues have been isolated. Among them, 14 short-chain peptides are associated with the K⁺ channel toxin family, 51 long-chain peptides are related to the Na⁺ channel toxin family and only one long-chain peptide is identified as a blocker of voltage-dependent K⁺ channels (Kᵥ) (2).

In our previous paper, a systematical isolation has been achieved and 11 short-chain peptides have been characterized from the venom of Chinese scorpion BmK (13) and solution structures of some short-chain scorpion toxins (less than 40 amino-acid residues long) have been described (14-16). In the present work, we report purification, characterization and sequence determination of a novel BK potassium channel blocker BmP09, which is composed of a long-chain (66 amino-acid residues long) with four disulfide bridges. Assaying the effects of BmP09 on voltage-gated channels in MACCs and on BK channels (mSlo) expressed in Xenopus oocytes, we found that BmP09 was a better specific blocker of BK channels encoded by mSlo α subunits with perfect reversibility, in other words, it only took less than 5 seconds for a full recovery. Compared with the Charybdotoxin (ChTX), the superior selectivity and reversibility makes BmP09 a better tool for structural and functional studies on BK channels.
EXPERIMENTAL PROCEDURES

Purification and chemical characterization of toxin BmP09

Crude venom was collected by electrical stimulation of the telson of scorpion BmK bred in captivity in Henan Province, China. The peptide was purified as described previously (13): lyophilized crude venom was dissolved in NH₄HCO₃ buffer (50 mM, pH 8.5) and centrifuged at 4000 g for 15 min. The supernatant was loaded onto a Sephadex G-50 column (2.5 × 150 cm, Pharmacia Fine Chemicals), which was equilibrated and eluted with the same buffer (Fig. 1A). The fraction III from the Sephadex G-50 column was loaded onto a Mono S cation exchange column (HR 5/5, Pharmacia LKB Biotech., Inc.), eluted with a step gradient of solution A to solution B at pH 5.0 (Fig. 1B). Solution A contained NaAc (20 mM), and solution B contained NaAc (20 mM) and NaCl (1 M). It was followed by similar separation on another Sephadex G-50 column (Fig. 1C). The final purification of BmP09 (G3512) was performed by using a reverse-phase HPLC column (C₁₈ column, 4.6 × 250 mm, 5 mm bead size, Alltech) eluted with a linear gradient from solution C to 50% solution D in solution C at a flow of 1 ml/min. Solution C contained CH₃CN (10%) and trifluoroacetic acid (0.1%) in H₂O, and solution D contained H₂O (20%) and trifluoroacetic acid (0.1%) in CH₃CN (Fig. 1D).

The molecular weight of BmP09 was measured using a LCMS-2010A ESI-MS instrument (Shimadzu, Japan). Amino acid analysis was performed on a Beckman 6300 apparatus (Beckman, USA) after hydrolysis of the sample in HCl (6 M) under vacuum at 110 °C for 18 h. The N-terminal sequence of BmP09 was achieved by Edman degradation using a Beckman LF3200 Protein-Peptide sequencer.
**Primary sequence determination of BmP09**

Because of the existences of disulfide bonds in the sequence, the sample of BmP09 was subjected to DTT reduction and iodoacetamide derivatization before MS analysis. Peptide BmP09 was reduced with a 250-fold molar excess of DTT in 0.25 M Tris-HCl buffer (pH 8.5) containing 6 M guanidine HCl and 4 mM EDTA. Reduction was carried out in the dark under nitrogen at 37 ºC for 1 h. Free thiols were alkylated by addition of a 500-fold molar excess of iodoacetamide held at room temperature for 30 min in the dark. The sample was load onto a 10% Tris-Tricine gel for SDS-PAGE to remove reagents. Peptide mapping study was performed on S-alkylated BmP09 as the TPCK-trypsin took place *in situ* in the gel after electrophoresis. AutoMS-Fit Automation Software was used to analyze the masses of peptide fragments obtained by digestion.

Sample of BmP09 (100 µL, 50 ng/µL) was digested with carboxypeptidase P and Y (Sigma, USA, 0.2 ng/µL, enzyme/substrate ratio was 1/500, w/w) in aqueous solution at 25 ºC. Every 6.0 µL digested sample was taken out at 20 s, 40 s, 60 s, 2 min, 5 min, 10 min, 15 min, 20 min, 30 min, 60 min, 2 h and 4 h, after the onset of incubation and each aliquot was acidified with 1% TFA and lyophilized, immediately. Each lyophilized sample was mixed at the ratio of 1:1 with a 4 mg/mL solution of alpha-cyano-4-hydroxycinnamic acid (CH₃OH:H₂O, 0.1% TFA) and performed by MALDI-TOF MS spectrum using a Voyager-DE STR (Applied Biosystems, USA) operated in the reflection mode with time lag focusing.

**Electrophysiology and Solutions**

**Chromaffin cell preparation**

Based on several early studies, mouse adrenal chromaffin cells (MACCs) were isolated and maintained as described previously (17, 18). In the patch-clamp experiments to compare effects of
BmP09 and BmK AS-1, we used rat adrenal chromaffin cells (3, 16, 19). Dispersion of Chromaffin cells was typically done on adrenal medullas from two or three Wistar mice (20 ~ 30 g) ~1 month age. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) in a standard CO₂ incubator at 37 °C, and currents were recorded 1 ~ 5 days after plating.

**Mutagenesis**

Point mutations of mslo, F266L and F266A, were produced by using QuikChange protocol (Stratagene). In brief, PCR reactions were performed by using the wild-type mslo as a template and a pair of complementary mutagenesis primers (F266L: 5'-CAGGGGACCCATGGGAAAA TCTTCAAAAACAACCAGGCACCTAC-3' and 5'-GTAAGTGCCTGGTTGTTTTGAAGATTTT CCCATGGGTCCCTG-3'; F266A: 5'-CAGGGGACCCATGGGAAAATGCTCAAAACAACC AGGCACCTACG-3' and 5'-CGTAAGTGCCTGGTTGTTTTGAGCATTTTCCCATGGGTCCCTG-3'). The PCR reaction mixture was then cut with the enzyme DpnI to digest the template wild-type mslo. After DpnI digestion, the PCR product was transformed into competent bacterial cells to amplify the mutant plasmid of mslo. All mutant constructs were verified by sequencing.

**Expression in Xenopus Oocytes**

Methods of expression in Stage V-VI Xenopus oocytes were described as previously described (7). Oocytes were defolliculated by treatment with 2 mg/ml collagenase I (Sigma-Aldrich Corp., St. Louis, MO) in zero-calcium ND 96 solution. Between 2 and 24 h after defolliculation, 1~2 ng of α (mslo) cRNA (a gift of Dr. Christopher Lingle, Washington Univ., St. Louis, MO) were injected into Xenopus oocytes using a Drummond Nanoject II (Drummond Scientific Co., USA). After injection, Oocytes were then incubated in ND-96 solution at 18 °C. Currents were recorded 2 ~ 7 days after RNA injection. ND-96 solution (pH 7.5) containing
concentrations of (in mM): 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 2.5 Na pyruvate, and 10 H⁺-HEPES, supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (only for incubation).

**Solutions**

For MACCs, the normal extracellular solution contained the following (in mM): 150 NaCl, 5.4 KCl, 1.8 CaCl₂, 2 MgCl₂, and 10 H⁺-HEPES (pH 7.4) titrated with NaOH. For whole-cell recording, the standard pipette solution contained (in mM): 130 K-glutamine, 30 KCl, 0.1 EGTA, 10 H⁺-HEPES, 0.05 GTP and 2 MgATP (pH 7.4). The “high TEA (tetraethylammonium chloride)” solution was the same as the standard bath solution except that the 20 mM NaCl was replaced by 20 mM TEA. The “high Cs⁺” solution contained the following (in mM): 130 Cs-glutamine, 30 CsCl, 0.1 EGTA, 10 H⁺-HEPES, 0.05 GTP and 2 MgATP (pH 7.4).

To record Na⁺ currents, the “high TEA” solution was used as bath solution, and pipettes were backfilled with “high Cs⁺” solution. For Ca²⁺ current recording solutions, the bath solution contained (in mM): 160 TEA, 5 BaCl₂, 10 H⁺-HEPES, and 0.1 EGTA, with pH adjusted to 7.4 with tetraethylammonium hydroxide and pipette solution was “high Cs⁺” solution. For oocytes, during recordings, oocytes were bathed in the solution contained (in mM): 160 MeSO₃K, 10 H⁺-HEPES, and 2 MgCl₂, adjusted to pH 7.0 with MeSO₃H. Pipettes were filled with a solution containing (in mM): 160 MeSO₃K, 10 H⁺-HEPES, and 5 HEDTA with added Ca²⁺ to make 10 μM free Ca²⁺, as defined by the EGTAETC program (E. McCleskey, Vollum Institute), with pH adjusted to 7.0. All of the chemicals were obtained from Sigma.

**Patch-clamp recording from single cells**

Patch pipettes pulled from borosilicate glass capillaries had resistances of 2 ~ 6 MΩ when
filled with internal solution. An outside-out patch was obtained by excising the patch from a cell in the whole cell configuration. Experiments were done and recorded using an EPC-9 patch clamp amplifier and PULSE software (HEKA Electronics, Germany). Currents were typically digitized at 20 kHz. Macroscopic records were filtered at 2.9 kHz during digitization. Single-channel records were filtered at 10 kHz.

During recording, drugs and control/wash solutions were puffed locally onto the cell via a puffer pipette containing seven solution channels. The tip (300 µm diameter) of the puffer pipette was located about 120 µm from the cell. As determined by the conductance tests, the solution around a cell under study was fully controlled by the application solution with a flow rate of 100 µl/min or greater. All pharmacological experiments met this criterion. All these experiments were done at room temperature (22 ~ 25 ºC).

Data analysis

Data were analyzed with IGOR (Wavemetrics, Lake Oswego, OR, USA), Clampfit (Axon Instruments, Inc. USA), SigmaPlot (SPSS Inc. USA), and QUB (SUNY, Buffalo, NY) softwares. Unless stated otherwise, the data are presented as means ± S.E.M., significance was tested by Student’s T test, and differences in the mean values were considered significant at probability < 0.05.

Dose-response curve for the percent block of BK currents was drawn according to a Hill equation $I = I_m/(1 + ([\text{toxin}]/EC_{50})^n)$, where $I_m$ is maximum blocking percentage of BK currents, and [toxin] is the concentration of BmP09. $EC_{50}$ and $n$ denote the toxin concentration of half maximal effect and the Hill coefficient, respectively.

Homology modeling and docking experiment
The model of BK channel (20) was generated by homology modeling on the basis of the crystal structure of the bacterial KcsA channel (PDB code 1BL8) (21), using the software SYBYL6.3 (Tripos Associates, 1996). The sequence alignment between KcsA and BK channel was obtained using the same criteria as those described by Gao and Garcia (22). The homology model of BK channel was further subjected to Powell minimization (2000 steps) using Kollman force field.

The models of toxin BmP09 and BmK AS-1 (23) were generated by homology modeling on the basis of the crystal structure of the toxin Neurotoxin 2 (PDB code 1JZB) (24). The sequence alignment between them was described as Fig. 3. The homology models of toxin BmP09 and BmK AS-1 were further subjected to energy minimization and dynamic simulation.

The surface electrostatic distribution analysis indicated that BmP09 preferred association with the entryway of K⁺ channel using the positively charged patch with the side chain of Lys41 in the center. The program “O” (version 8.0.6) (25) was used for the docking experiment. BmP09 was docked manually into the outer of entryway of the BK channel model along with the dipole direction. As expected, the mouth of the K⁺ channel bears a large negative charge, whereas the surface of the toxin BmP09 has a positive charge. The electrostatic potential between the toxin BmP09 and the K⁺ channel attracted the positively charged toxin to the entryway of the channel. In order to obtain favorable toxin-channel clusters, the toxin molecule was allowed to rotate during the docking process. The most stable cluster with the best fitting between the toxin and the K⁺ channel was used to analyze the contacts between the BmP09 and the BK channel.

The BmP09/BK channel cluster docked most favorably was further subjected to energy minimization for 2000 steps to achieve the gradient tolerance 0.05 kcal/(mol Å) using the Powell
algorithm and the Kollman force field in the software SYBYL6.3. Molecular dynamics simulation using the Powell algorithm was then carried out for the complex for 100 fs at 300 K. Kollman force field constraints were applied on the backbones of the channel in the region comprising residues His254 to Val278, while the remaining part of the channel was kept fixed during the simulation. The structure of the peptide was completely unconstrained. A cutoff distance of 8 Å was used for non-bounded interactions. An integration time-step of 0.1 fs was used and coordinate sets of the trajectory were saved every 2 fs. Every structure obtained from the coordinate sets over the 100 fs of simulation was performed with 500 steps of minimization. Finally, the average structure was energy minimized with 1000 steps of Powell minimization.
RESULTS

Purification of BmP09

The crude venom was initially separated into four fractions (I–IV) by gel-filtration chromatography on a Sephadex G-50 column (Fig. 1A). Separation of the fraction III on a Mono S cation exchange column gave 5 fractions (Fig. 1B). Among the 5 fractions, the fraction 5 was further separated on another Sephadex G-50 column, and two sub-fractions 351 and 353 were obtained (Fig. 1C). A pure peptide was obtained after the separation of fraction 351 on a reverse-phase HPLC column (Fig. 1D).

Primary sequence of BmP09

The molecular weight of BmP09 was 7721 Da, as determined by ESI MS (Fig. 1E). The results of amino acid analysis (Table 1), N-terminal 14 residues sequence analysis (DNGYL LNKYT GCKI) and peptide-mapping studies (Fig. 2) are consistent with those calculated from the mature peptide BmK AS-1 derived from cDNA (AF079061, EMBL). The difference in molecular weight between the ESI-MS data (7721 Da) and calculated value (7704.8 Da) according to the sequence of BmK AS-1 could be attributed to the oxidation of C-terminal Met residue (26). The oxidative modification could be validated by the MS analysis of the carboxypeptidase-digested products. Actually, a principal ion with the MS value of 7575 Da was observed under the molecular ion in the MS spectrum of the carboxypeptidase-digested products. The mass difference (147 Da) is well accounted for a Met residue with a sulfoxide group. Therefore, the sequence of BmP09 is the same to that of BmK AS-1, and only differs at the C-terminus by an oxidative modification (see Figure 3).

Effects of BmP09 on Voltage-gated Channels in Chromaffin Cells
Mouse adrenal chromaffin cells (MACCs) are excitable cells and express variety of voltage-gated channels such as voltage-gated Na+, K+, and Ca2+ channels. They are widely used as a neuronal model for studying the features of channel behaviors and searching the targets of toxins (27-31). To study whether the BmP09 affects voltage-gated ion channels, we started to screen for its effects on the MACCs. As shown in Fig. 4A, whole cell currents were elicited by 60 ms voltage ramps from -90 mV to +100 mV within the normal extracellular solution in the presence of and in the absence of 100 nM BmP09. With the augment of membrane potential by the voltage ramp, inward currents of Na+ and Ca2+ channels first emerged at ~0 mV and then outward currents of voltage-gated K+ channels including both the KV and the KCa channels started to step up gradually (28, 31). In Fig. 4A, a trace in dark line shows a 67% reduction of outward maximum currents, by the application of 100 nM BmP09, with a slightly increase on inward currents. The reduction of outward currents may result in the slightly increase of inward currents. In addition, after removal of BmP09, the current trace indicated in Fig. 4A is almost back to the control level, which hints that the blocking behavior of BmP09 is reversible.

Voltage-gated sodium channels play a critical role in repeatedly firing of action potentials and propagation in excitable cells (2). Most of long chain peptides have been proved to be the blockers of Na+ channels such as BmK AS-1 (32). However, the traces in Fig. 4B, activated by voltage steps to 0 mV after a prepulse to −90 mV to remove inactivation, are overlaid to emphasize that there is no inhibition on Na+ channels before, during and after the application of the toxin 100 nM BmP09. In contrast, BmK AS-1 has inhibitive effect on Na+ channel in chromaffin cells (32), but no effect on the K+ currents (n = 5, data not shown). In chromaffin cells, there exist most types of calcium channels, of which L-type calcium channel is clustered with BK channels (33).
were three possibilities for the inhibition of BmP09 on outward K⁺ currents, the first one was directly blocking on Ca²⁺-activated K⁺ currents, the second was directly blocking Kv⁺ channels and the third was indirectly blocking on Ca²⁺ channels itself. Therefore, we intended to verify whether BmP09 blocked voltage-gated calcium channels first. In Fig. 4C, Ca²⁺ currents were elicited by 200 ms depolarization from holding potential −70 mV to 0 mV. Three traces of Ca²⁺ currents are nearly at the same level for 0, 100 and 0 nM BmP09, which suggests that BmP09 has negligible effects on the inward Ca²⁺ currents. This result also hinted that the BmP09 blocked Kv⁺ /BK channels.

Selectivity of BmP09 among K⁺ channels

K⁺ selective channels with a tremendous diversity are found in probably all the cells. K⁺ channels, when they are open, set the resting potential, keep fast action potential short and so on (34). However, we often do not know which types are present in cells, e.g. in MACCs. As we know, there are at least two types of Kv channels, i.e. a delayed-rectified channel and an A-type transient channel, and two types of Ca²⁺-dependent K⁺ channels (KCa), i.e. a small conductance Ca²⁺-dependent K⁺ channel (SK) and a BK channel, in MACCs. It is hard to distinguish their individual types in MACCs, but is easy to separate Kv and KCa channels by applications with alternating calcium concentration in the bath solution. In the Fig. 5A and 5B, all traces were activated by 100 ms depolarization to +80 mV from holding potential of −70 mV, which was designed to avoid the calcium influx induced by the opening of calcium channels at ~0 mV. In Fig. 5A, 100 nM BmP09 obviously blocked the “Kv” currents within the normal bath solution, i.e. 1.8 mM Ca²⁺ in the normal saline. But, applied with Ca²⁺-free normal saline at the above patch, we found that 100 nM BmP09 had no effect on the remaining currents as shown in Fig. 5B. Now we
think that traces in Fig. 5B present \( K_V \) currents only. This also means that the protocol as indicated in Fig. 5B cannot completely avoid calcium influx into MACCs for an unknown reason. In all experiments, 20 mM TEA in Fig. 5A and 5B was applied extracellularly for subtracting leak currents.

Small-conductance Ca-activated \( K^+ \) channels (SK channels) play an important role in modulating excitability in MACCs. SK channels are voltage-independent and activated by submicromolar concentration of intracellular calcium (35). Based on the results shown in Fig. 4 and Fig. 5A and 5B, we knew that currents inhibited by BmP09 were \( K_{Ca} \) channels. Now we need to identify which channel of \( K_{Ca} \), i.e. SK or BK, blocked by the BmP09. In Fig. 5C, SK currents were activated by a test pulse to \(-100\) mV after the 100 ms prepulse to 0 mV to upload calcium ions into cytosolic membrane. In each patch, 200 nM apamin was used to identify the toxin-sensitive components. It is clearly that the BmP09 has little effect on the SK currents based on traces showed in the inset of the Fig. 5C. To investigate the detailed blocking effects on BK currents by the BmP09, we adopted Xenopus oocytes as an expression system instead of MACCs. In Fig. 6A, currents from an outside-out macropatch with 10 \( \mu M \) \( Ca^{2+} \) in the pipette were elicited by a voltage protocol indicated at the top panel. In each patch, 20 mM TEA was applied extracellularly to obtain remaining leak currents. Subtracting leak currents, we found that 100 nM BmP09 blocked \(~80\%\) (Fig. 6A). On an average, BK currents were reduced by \( 76.1\% \pm 8.5\% \), with applying 100 nM BmP09 (Fig. 6B). The \( EC_{50} \) of BmP09 on \( BK_{Ca} \) channels was assessed to be \( 27\) nM with a Hill coefficient \( n = 1.8 \), according to the dose-response curve fitting (Fig. 6C). BmP09 also blocked the inactivating currents of mslo1/\( \beta_2 \) coexpressed in Xenopus oocytes with an over 100 nM \( EC_{50} \) (data not shown).
In comparison with the scorpion toxin ChTX, an antagonist of BK channels, the time course of BmP09 blocking BK channels was undergoing in Fig. 7. For BmP09 (Fig. 7A), both the onset and offset of blockings are very rapid with a complete recovery. The blocking behavior of BmP09 on BK channels is similar to the TEA blocking K⁺ channels. Correspondingly, the time course of 100 nM ChTX (Fig. 7B) shows a much slower onset and offset on BK currents with only 80% recovery.

Single channel recordings (Fig. 8), in an outside-out patch with 10 μM Ca²⁺ in the pipette, derived from BK channels encoding with mSlo1 α subunit expressed in Xenopus oocytes. Currents were activated every 3 s by depolarizations to 40 mV from a holding potential –140 mV for 500 ms. Figure 8A shows that the representative sweeps and the ensemble-average traces of 20 continuous sweeps in the absence or presence of BmP09, respectively. The corresponding amplitude histograms in Fig. 8B are shown under the traces in Fig. 8A. Statistical analysis revealed that BmP09 had no effect on single BK channel conductance (~250 pS under symmetrical 160 mM K⁺ solutions), but reduced the probability of being open or increased the close time of the single BK channel. The total open time of the single BK channel was decreased to almost 30% over the control during applications of 100 nM BmP09. These values were consistent to those of macroscopic BK currents.

**Molecular modeling of BmP09**

As shown in Fig. 9A, the structure of BmP09 model has the common characteristic of α-type toxin. It contains one α-helix (residues 22-29), and a three-strand anti-parallel β-sheet (residues 2-4, 34-37, and 44-47). The α-helix is connected to the middle strand of the β-sheet by a pair of disulfide bonds involving Cys23-Cys44 and Cys27-Cys46. The longer outer strand of the β-sheet...
is linked to the long loop prior to the α-helix by a disulfide bond between Cys16 and Cys37. The fourth disulfide bond between Cys12 and Cys62 limits the flexibility of the C terminus. There is an additional shorter two-strand anti-parallel β-sheet formed by residues 6-7 and residues 12-13 of BmP09, compared with Neurotoxin 2.

The surface electrostatic charge distribution of BmP09 is shown in Fig. 9B. It contains a dense positively charged region mainly composed of the basic residues Lys13, Lys41 and Lys65. The Lys41 is located at the loop between the middle strand and outer strand of the β-sheet; the Lys65 is located at the C terminus, while the Lys13 is partly buried. The specificity of scorpion toxin for the various potassium channels has been extensively investigated. The results revealed that binding of the peptide is governed by electrostatic interactions between negatively charged residues in the channel and positively charged residues in the peptide (36, 37). The surface electrostatic distribution shown in Fig. 9B indicated that BmP09 preferred association with the entryway of the BK channel using its positive patch around the side chains of Lys13, Lys41 and Lys65.

The structure of BmP09/BK channel cluster with the favorable electrostatic energy was further refined and the optimized structure of BmP09/BK channel complex is shown in Fig. 9E. The principal interactions between the toxin BmP09 and the BK channel derived from the refined complex structure were analyzed using the LIGPLOT program (38), and the results are summarized in Table 2. Four hydrogen bonds and three hydrophobic contacts existed in the refined complex. Therefore, the interface between the BmP09 and the BK channel is large and involves about seven residues of BmP09 and twelve residues of the BK channel, respectively.
DISCUSSION

In the present study, we described the structure and function of the novel scorpion toxin BmP09. We found that: (1) the amino acid sequence of BmP09 was identical to the BmK AS-1 except a sulfoxide Met66 residue at the last C-terminus in BmP09; (2) In contrast to BmK AS-1, 100 nM BmP09 selectively blocked BK channels with no effect on Na⁺ channels based on the results from Chromaffin cells; (3) BmP09 reduced the open probability of the reconstituted BK channels encoded with mslo1 but not altering the single-channel conductance; (4) A mechanism of BmP09 blocking mSlo1 channels was proposed by simulating the ligand/channel binding, based on molecular structures of BmP09 and mSlo1.

The features of BmP09 in blocking BK channels

In this study, we have described the purification, characterization, and electrophysiological behavior of BmP09. The sequence of BmP09 is almost identical to that of the known toxin BmK AS-1 with an only difference at the C-terminus by an oxidative modification (23, 39). The Met66 with a sulfoxide group makes BmP09 a dramatic different function compared with BmK AS-1. BmK AS-1 blocks the Na⁺ channel with no effect on K⁺ channels, while BmP09 shows completely opposite results. Upon probing the targets of BmP09 toxin on chromaffin cells, we found that 100 nM BmP09 inhibited BK currents without affecting other voltage-gated Na⁺, K⁺, and Ca²⁺ channels in MACCs. This was further confirmed by blocking directly the BK channels expressed with mSlo1 α-subunits in Xenopus oocytes. It is well known that most of long-chain scorpion toxins are reported as blockers of Na⁺ channels. One long-chain scorpion toxin TsTxKβ was only found a blocker of voltage-gated non-inactivating K⁺ channel (40). To our knowledge, this is the first report regarding a long-chain peptide as a specific blocker of BK channels.
Furthermore, the toxin BmP09 exhibited perfect reversibility in blocking BK channels compared with the Charybdotoxin (ChTX). As shown in Fig. 7A, both the onset and offset during BmP09 blocking BK channels were very rapid (less than 5 s) and the recovery was complete. In comparison with ChTX, the offset course of 100 nM ChTX was extremely slow (more than 100 s) and incomplete, as shown Fig. 7B. The BmP09 blocking BK currents is very similar to the TEA blocking K⁺ channels.

On the other hand, BK channels can be formed by α-subunit alone or by α-subunit bound with up to four β subunits. Co-expression with α and β subunits, of which the latter, composed of two transmembrane domains with a long extracellular loop, modifies the kinetic behaviors of BK currents encoded with α subunit alone, such as Ca²⁺ sensitivity and pharmacological properties and so on. Typically, it will reduce the sensitivity to scorpion toxins (6). Actually, the N-linked glycosylation of β subunits excluding β₁ plays a role in increasing the EC₅₀ of toxins on Slo1 alone to more than twenty fold (41, 42). As compared with ChTX or IbTx, the toxin BmP09 did not show any notable difference on blocking the inactivated currents co-expressed with the mSlo1 α and β₂ subunits.

**The difference in the solution conformations of BmP09 and BmK AS-1**

As demonstrated in previous section, the scorpion peptides BmP09 and BmK AS-1 possessing high homology in their primary sequences showed remarkable differences in their specificities towards Na⁺ and K⁺ channels. These distinctions may be related to their 3D structures. To differentiate the structural features of these two toxins, we generated their 3D-structural models by homology modeling. As shown in Figure 9 (C-D), these two peptides possessed the same global folding and differed clearly in the conformations of their C-terminal segments. For BmK
AS-1, the C-terminal residue Met66 extends to the hydrophobic center of the molecule as a result of its hydrophobicity to form a sulfur-π interaction (43), and its carboxylic group forms a salt bridge with the side chain of the basic residue Lys41. In BmP09, the C-terminal residue Met66 with a sulfoxide side chain turns back due to its less hydrophobicity, of which the side chain forms a hydrogen bond with the basic side chain of Lys13 residue. Therefore, the properties of the residue Lys41 in these two peptides are obviously conflict. In BmK AS-1, the residue Lys41 is less basic for the salt bridge and is partly buried in the hydrophobic center, while the basic side chain of Lys41 residue in BmP09 is free and is exposed to the surface of the molecule. These differentiations should be responsible for their distinct behaviors on Na⁺ and K⁺ channels.

**The possible interaction mode of BmP09 with BK channel**

The mechanism underlying the blockade of voltage-gated K⁺ channels by α-KTx toxins have been intensively explored by the modeling analysis of toxin/K⁺ channel complex generated by docking and dynamic simulation in the recent decade (11, 44-46). In order to elucidate the possible interaction mode, the 3D structural model of BmP09/BK channel complex was generated by docking and dynamic simulation (Fig. 9E). In Figure 9E, the positively charged side chain of Lys41 lies in the center of the peptide/channel interface to form hydrogen bonds with the four backbone carbonyl groups of Tyr290. In addition, Asn2, Tyr36, Tyr57 and Lys65 form hydrogen bonds with the residues Asn269 (IV), Asn268 (IV), Lys296 (I) and Gln270 (I) of the BK channel, respectively. Meanwhile, three aromatic interactions were identified: Phe39 of BmP09 forms favorable aromatic contacts with Phe266 (III) of the BK channel, while Tyr34 and Tyr57 of BmP09 form aromatic contacts with Phe266 (IV) and Phe266 (I), respectively. Therefore, the interaction mode of BmP09 features that the Lys41 is in the center as a pore blocker, which is
surrounded by a network of hydrogen bonds and aromatic π-π interactions between the peptide and the BK channel. On the basis of simulation, F266 plays an important role in stabilizing the binding of the BmP09 and the BK channel. To confirm the interaction between mSlo1 and BmP09, we mutated the residue Phe266 of mSlo1 to Alanine or Leucine. Even though the currents of F266A/L elucidated by the same protocol shown in Fig 6A were still blocked by 100 nM BmP09 (Fig. 10A), however, the percentage of blockade for F266A and F266L was reduced to less than 20% (Fig. 10B).

**Comparison of the interaction mode between CTX/BK channel and BmP09/BK channel complex**

The interaction mode of CTX peptide with BK channel has been investigated in details by analysis of the complex model derived from the docking and dynamic simulation (22). To rationalize the difference in the reversibility in blocking BK channels between CTX toxin and BmP09, a comparison of the interaction modes of CTX/BK and BmP09/BK channel complexes has been made, and the results were summarized in Table 3. For the model of CTX/BK complex, the central residue K27 is positioned at the center and its positively charged side-chain hydrogen bonds with four backbone carbonyl oxygen atoms of Y290 in the selectivity filter. Besides, two salt bridges, two hydrogen bonds as well as three aromatic contacts make favorable contribution to the binding. For BmP09/BK channel complex, besides basic residue K41, which lies in the center of the peptide/channel interface to form hydrogen bonds with the four backbone carbonyl groups of Y290, four hydrogen bonds and three aromatic contacts constitute the primary interactions. However, the distribution of these hydrogen bonds and aromatic interactions around the central residue in these two complexes is quite different. In CTX/BK channel complex most hydrogen
bonds, salt bridges and aromatic interactions lie in the inner of the interface and close to the central residue (within about 6–7 Å), only Asn30 is about 9 Å apart from the central residue (ref. Table 3). Since CTX is a globular structure with a spherical surface, those inner hydrogen bonds and aromatic interactions close to the central residue should be buried deeply, only Asn30 lies at the outer edge of the interface. In contrary, in BmP09/BK channel complex most hydrogen bonds and aromatic interactions are apart from the central residue (larger than 9 Å) and locate at the outer edge of the interface of the complex as shown in Figure 9F. These facts could well account for the difference in the reversibility in blocking BK channels between CTX toxin and BmP09.

For CTX/BK channel complex, both association and dissociation rates should be slower due to a number of inner and buried hydrogen bonds, salt bridges and aromatic interactions. In comparison, those of BmP09/BK channel complex must be quicker because its hydrogen bonds and aromatic interactions locate at the outer edge of the interface and are exposed to the solvents.

On the other hands, the time scale of association and dissociation can be further evaluated on the basis of the $K_d$ data. The time scale of a toxin-receptor reaction is set by a formula $K_d = k_{-1}/k_1$, where $k_1$ is the second-order rate constant for binding ($M^{-1}s^{-1}$), $k_{-1}$ the first-order rate constant for unbinding ($s^{-1}$), and $K_d$ the equilibrium dissociation constant (M) of the toxin-receptor complex (47). Meanwhile the time constant for unbinding $\tau_{unbinding} = 1/ k_{-1}$. Now we use the above formula to estimate the time scale of unbinding for toxin BmP09. Assuming that $\tau_{unbinding}$ of CTX-BK complex is set to 200 s, and $K_d = 4$ nM (7), we have $k_{-1} = 1/(\tau_{unbinding} * K_d) = 1.25 \times 10^6 M^{-1}s^{-1}$.

Regarding the toxin BmP09, we assume $k_1 = 1.25 \times 10^7 M^{-1}s^{-1}$, because the time scale of BmP09 for binding to BK channels is ten-fold fast than the one of CTX (Fig. 7). Considering the $K_d = 27$ nM for BmP09, the unbinding time constant for BmP09 is simply obtained to be about 3 s,
which is well consistent with the experimental data in this study.

Altogether, BmP09 is the first long-chain scorpion peptide as a specific and reversible blocker of BK channels. The structural information derived from the modeling and docking analysis may be helpful in designing specific inhibitors of BK channels.
Acknowledgements

We thank Dr. Yong-Hua Ji for gift of BmK AS-1. We also thank Prof. T. A. Jones of Uppsala University, Sweden, for the software program “O” and Tripos, Inc., for the Sybyl6.3 software package. This work was supported by Grants from the National Science Foundation of China (20132030, 30025023, 30470445, 3000062, 30130230, 30328013 and 30330210), the Major State Basic Research Program of P.R. China (G1999054000, 2001CCA04100, and G2000077800), the Li Foundation and the Sinogerman Scientific Center, and Chinese Academy of Sciences (KGCX2-SW-213-05).
REFERENCES


*Biochemistry* **43**, 12469-12476


FIGURE LEGENDS

Figure 1 Isolation and purification of BmP09.


Figure 2 Peptide-mapping for the fragments.

Peptide-mapping comes from TPCK-trypsin digestion of the S-alkylated BmP09. The Met residue labeled with an asterisk (*) represents an oxidative modification.

Figure 3 Sequences alignment of BmP09 with other long-chain scorpion toxins.

The sequences of BmK AS-1 (23), Neurotoxin 1 (48), Neurotoxin 2 (48) and BmK I (49) were obtained from EMBL with accession numbers: AF079061, AF338454, AF338453, and AF057554. Sequences alignments have been performed according to their cystein residues and gaps are presented as dashes. Here, the M* represents the sulfoxide Methionine.

Figure 4 Effects of the scorpion toxin BmP09 on K⁺, Na⁺ and Ca²⁺ currents in MACCS.

A, Whole-cell currents were elicited by a 60 ms voltage ramp from -90 mV to +100 mV in the normal extracellular bath solution. The 100 nM BmP09 reduced the outward K⁺ current around 67% in this cell. B, Fast inward Na⁺ currents were activated by a voltage step to 0 mV, after a
prepulse to −90 mV to remove inactivation, in the bath solution with 20 mM TEA and 160 mM Cs+ internal solution. A trace obtained at 100 nM BmP09 is nearly the same as in control (n = 6).

C, Ba2+ currents were elicited at 0 mV from a holding potential of −70 mV in 160 mM TEACl and 5 mM BaCl2 bath solution. The pipette was backfilled with 160 mM Cs+ solution. At 100 nM, BmP09 results in negligible effects on the inward Ba2+ current (n = 5).

Figure 5 BmP09 has no effect on Ca2+-independent, but voltage-dependent K+ currents, and has little effect on SK currents.

A, In 1.8 mM Ca2+ bath, the BmP09 partially reduced the K+ current (n = 10), in which a Ca2+-dependent component should be involved. B, In Ca2+-free bath solution, the traces of K+ currents, activated by a voltage protocol indicated at the bottom, show that BmP09 has no effect on the K+ current (n = 10). In both cases, 20 mM TEA was applied in all experiments to obtain net K+ currents. This means that the toxin BmP09 is exclusively sensitive to Ca2+-dependent K+ currents. C, In 1.8 mM Ca2+ bath solution, SK currents were activated at −100 mV by the Ca2+ influx during 100 ms prepulse to 0 mV from the holding potential −70 mV. A voltage protocol is shown at the top panel. At the bottom panel, 200 nM apamin was applied to show SK currents in all the patches and currents in the patch were all in the same level for 0 nM, 100 nM and 0 nM BmP09 solutions (n = 15), which suggested that BmP09 had no effect on SK currents.

Figure 6 BmP09 blocks currents of mslo1 α subunit expressed in Xenopus oocytes.

A, Traces show the BK currents from an outside-out patch from a Xenopus oocyte injected with cRNA encoding mouse Slo1 α-subunit. Channels were activated by a voltage step to +100 mV,
after a prepulse to –180 mV, in the presence of 10 μM Ca²⁺. A voltage protocol is shown at the top panel. BK currents encoded with mSlo1 were remarkably reduced by 100 nM BmP09. B, on an average, 100 nM BmP09 reduced BK currents by 76.1% ± 8.5% (p < 0.01, n = 20, student’s t-test). 20 mM TEA was applied before applications of the toxin BmP09 for subtracting leak or native currents. C, The dose-response curve of BmP09 blocking BK currents was fitted by Hill equation (see “experimental procedures”). The EC₅₀ value is 27 nM, and the Hill coefficient is 1.8 (n = 5).

Figure 7 The time course of BmP09 blocking BK currents.
A, Normalized peak current amplitudes from patches shown above are plotted as a function of elapsed time. The patch was perfused with 20 mM TEA and 100 nM BmP09 indicated by the horizontal bars, respectively. Fits to the onset and offset time courses of BmP09/BK give \( \tau_{on} = 2 \) s and \( \tau_{off} = 1 \) s, respectively. Depolarizations were applied every 500 ms by the protocol indicated in Fig. 6A and, as a comparison, the normalized results for 100 nM ChTX are plotted. Fits to the onset and offset time courses of ChTX/BK give \( \tau_{on} = 20 \) s and \( \tau_{off} = 109 \) s, respectively.

Figure 8 Effects of the toxin BmP09 on single BK channels expressed in Xenopus oocytes.
A, Traces in the middle panel show single-channel openings of BK channels in an outside-out patch. The cytosolic side of membrane was faced with 10 μM Ca²⁺. Channels were activated, by voltage steps from –140 mV to +40 mV for 0.5 s, before, during, and after the application of 100 nM BmP09. Ensemble-average currents from 20 continuous sweeps are shown below the traces. B, The corresponding amplitude histograms show that the average amplitudes of currents, at 40 mV, are 10.1, 10.6 or 9.6 pA and the probabilities of being open are 69%, 22% or 57%, respectively.
Figure 9

A, MOLMOL representation of the structure of BmP09 model. Nt and Ct indicate N-terminus and C-terminus, respectively. Three disulfide bridges are shown as neons (yellow). B, Electrostatic potential surfaces of BmP09 is calculated by MOLMOL program. Positively charged residues are shown in blue and negatively charged residues are shown in red, respectively. C-D, 3D structures of the toxin BmK AS-1 (C) and the toxin BmP09 (D), residues Tyr4, Lys13, Tyr36, Tyr38, Phe39, Lys41, Tyr45, Tyr57, Lys65 and Met66 are labeled and their side-chains are shown, while the other residues are indicated with blue-colored α-carbon atoms. E, The front view of the BmP09/Kca1.1 channel complex generated by MOLMOL. The residues Asn2, Tyr34, Tyr36, Phe39, Lys41, Tyr57 and Lys65, which have formed hydrogen bonds and hydrophobic contacts with the residues of the channel, are marked. F. Interaction interface of BmP09 with BK channel. The key interactions pairs are indicated with black lines, while the interactions between K41 of BmP09 and Y290 (I-IV) of the BK channel are not shown for clearer views. Blue, yellow, green, white, and grey surfaces represent basic, sulfur-containing, polar, nonpolar, and aromatic residues, respectively. The red lines highlight the distance between the key residues and central K41 on the interface of BmP09.

Figure 10 F266 stabilizing the conformation of the BmP09/BK channel complex

A, BK currents expressed with cRNA encoding F266L/A were activated by the protocol same to Fig 6A, in the presence of 10 μM Ca^{2+}. The BK currents of F266L/A were slightly reduced by 100 nM BmP09. B, on average, the 100 nM BmP09 only reduced the currents of F266A (left) and F266L (right) by 19.5% ± 4.2% (n = 14) and 15% ± 3.8% (n = 14), respectively (p < 0.01,
student’s t-test). 20 mM TEA was applied before applications of the toxin BmP09 for subtracting leak or native currents.
Table 1 The amino acid compositions of BmP09

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Number of residues</th>
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<tr>
<td>Asx</td>
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<tr>
<td>Thr</td>
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</tr>
<tr>
<td>Ser</td>
<td>2.06(3)</td>
</tr>
<tr>
<td>Glx</td>
<td>5.57(5)</td>
</tr>
<tr>
<td>Gly</td>
<td>5.93(6)</td>
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<tr>
<td>Ala</td>
<td>3.06(3)</td>
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<tr>
<td>Val</td>
<td>1.56(1)</td>
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<tr>
<td>Met</td>
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<tr>
<td>Ile</td>
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<tr>
<td>Leu</td>
<td>5.02(5)</td>
</tr>
<tr>
<td>Tyr</td>
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<tr>
<td>Phe</td>
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<tr>
<td>Arg</td>
<td>2.04(2)</td>
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<td>Lys</td>
<td>6.57(7)</td>
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<tr>
<td>Pro</td>
<td>1</td>
</tr>
<tr>
<td>Trp</td>
<td>3</td>
</tr>
<tr>
<td>Cys-Cys</td>
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<td>MW (calculated)</td>
<td>7704.81</td>
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<td>MW (experimental)</td>
<td>7721</td>
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Table 2 Observed Interactions Between the BmP09 scorpion toxin and the BK channel in the BmP09-BK channel complex.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Atom</th>
<th>Residue&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Atom</th>
<th>Distance (Å)</th>
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<tr>
<td>Asn2</td>
<td>OD1</td>
<td>Asn269 (IV)</td>
<td>ND2</td>
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<td>OH</td>
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<td>Tyr290 (I)</td>
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<tr>
<td>Lys41</td>
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<td>Tyr290 (II)</td>
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<tr>
<td>Lys41</td>
<td>NZ</td>
<td>Tyr290 (III)</td>
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<td>Tyr57</td>
<td>OH</td>
<td>Lys296 (I)</td>
<td>NZ</td>
<td>2.91</td>
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<tr>
<td>Lys65</td>
<td>NZ</td>
<td>Gln270 (I)</td>
<td>OE1</td>
<td>2.83</td>
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<sup>a</sup> I, II, III and IV represent the four chains of the BK channel.
Table 3  The comparison of the interaction mode between CTX/BK and BmP09/BK channel complexes

<table>
<thead>
<tr>
<th>Residues of BmP09</th>
<th>Distance from central Lys41 residue (Å)</th>
<th>Interaction formed with BK channel</th>
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<tr>
<td>Asn2</td>
<td>16.2</td>
<td>hydrogen bond</td>
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<td>Tyr34</td>
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<td>Phe39</td>
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<td>π-π interaction</td>
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<tr>
<td>Tyr57</td>
<td>13.2</td>
<td>π-π interaction and hydrogen bond</td>
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<tr>
<td>Lys65</td>
<td>11.5</td>
<td>hydrogen bond</td>
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<table>
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<td>Arg25</td>
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<td>Met29</td>
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<td>hydrophobic contact and sulfur-π Interaction</td>
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<td>Asn30</td>
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<td>hydrogen bond</td>
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<tr>
<td>Arg34</td>
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<td>salt bridge</td>
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<tr>
<td>Tyr36</td>
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Figure 1:
Figure 2:

Sequence of BmP09

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<td>DNGYLLNRYTNGCK</td>
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<tr>
<td>(1546.6801)</td>
<td>(1741.7121)</td>
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<tr>
<td>DNGYLLNRRNKH</td>
<td>INCVINNESCNECKLR (2009.8468)</td>
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<td>(936.44441)</td>
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<td>RGNYGYY Fus (1514.6519)</td>
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<td></td>
<td>GNYGYY Fus (1358.5942)</td>
</tr>
<tr>
<td></td>
<td>LACYCEGAPK (1168.4744)</td>
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<tr>
<td>Protein</td>
<td>Sequence</td>
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<tr>
<td>------------------------</td>
<td>--------------------------------------------------------------------------</td>
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<tr>
<td>EmF09</td>
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<tr>
<td>Emk AS-1</td>
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<td>Neurotoxin 2</td>
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<tr>
<td>Neurotoxin 1</td>
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<tr>
<td>EmX I</td>
<td>VRDVIKPHNYVEFAR------EYNDLITKNG---AKSGYQWVGKYGNGMHELPWNPF1RVPKG---</td>
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</table>
Figure 4:
Figure 5: 

A. 1.8 mM Ca$^{2+}$

B. 0 Ca$^{2+}$

C. -70 mV to -100 mV
Figure 6:

A

B

C

EC_{50} = 27 nM
Figure 7:
Figure 8:
Figure 9:
Figure 10:

A

0 mV 100 mV
-180 mV  120 mV

(F266A)

control
wash

0.5 nA
15 ms

Bmp09

TEA

(F266L)

control
wash

0.5 nA
15 ms

Bmp09

TEA

B

Normalized Amplitude

100

control  mslo  F266A  F266L

0  50