The existence of glycine residues in long-chain scorpion toxins has been well documented. However, their role as analgesics has not been evaluated. To address this issue, we investigated the functional role of glycines in the C-terminal end of Chinese-scorpion toxin from Buthus martensi Karsch (BmK AGP-SYPU2) using site-directed mutagenesis and analgesic activity assays. Recombinant BmK AGP-SYPU2 and its mutants were efficiently expressed in E. coli and purified to homogeneity using immobilized metal ion affinity chromatography (IMAC) and cation exchange chromatography. The mouse-twisting test was used to detect the analgesic activity of BmK AGP-SYPU2 and its mutants. As a result, we identified glycines at the C-terminal end that, when altered, significantly affected analgesic activity. Also, Mut6566 was significantly decreased compared to BmK AGP-SYPU2. These data indicate that the glycines at the C-terminal end are important for the analgesic activity of BmK AGP-SYPU2. [BMB reports 2010; 43(12): 801-806]

INTRODUCTION

Scorpion venoms are a rich source of neurotoxic proteins that modulate various ion channels, including Na⁺, K⁺, Ca²⁺ and Cl⁻, in excitable membranes (1). Scorpion long-chain neurotoxins (LCNs) are composed of 61-76 amino acid residues. Based on their mode of action and binding properties, LCNs affecting voltage-gated Na⁺ channels (VGSCs) are divided into two classes: α- and β-toxins. Scorpion α-toxins are further divided into three different pharmacological subfamilies according to their preferential activities against mammals and insects. These subfamilies include classical α-mammal toxins, insect α-toxins, and α-like toxins (2).

A better understanding of these toxins' structure-function relationships can be obtained by using site-directed mutagenesis.

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This method generates different mutants to study their interaction with the target channels (3, 4). It has been predicted that the N- and C-termini are important for the interaction of these toxins with their target channels (5).

Most LCNs share a common structural framework consisting of three double-stranded anti-parallel β-sheets linked to a short α-helix. The reverse turn in residues 8-12 and the C-terminal segment in residues 58-64 are closely connected by a fourth disulfide bond (Cys12-Cys63) to form a unique tertiary entity (reverse turn and C-terminal, Site RC) (6). This entity is believed to mediate a high-affinity interaction with the receptor (7). Extensive mutagenesis studies on this functional domain have successfully been carried out on scorpion α-toxins, such as Lqh αIT from Leirus quinquestriatus hebraeus (8-10) and BmK M1 from Buthus martensi Karsch (11-13). These studies shed some light on the structural and functional anatomy of scorpion α-toxins, which consist of three major functional domains. These studies also showed that some residues, particularly those found in the C-terminal region, are important for the formation of an electrostatic potential involved in receptor recognition and toxic activity. However, there has been no research on the contribution of C-terminal glycine residues on analgesic activity. A number of analgesic scorpion toxins have been identified and characterized from scorpion venom, but the structure-function relationship and the location of the functional site of these toxins still remain to be determined. In our previous studies, site-directed mutagenesis was applied to the conserved hydrophobic surface and disulfide bridges in an antitumor-analgesic peptide to determine the analgesic domain (14, 15). Our lab is interested in BmK AGP-SYPU2, which has been isolated and purified by ion exchange chromatography, hydrophobic interaction chromatography and gel filtration chromatography from the Buthus martensi Karsch (BmK) and was shown to have an analgesic effect at the animal level (data not shown). The sequence determination showed that the mature peptide of BmK AGP-SYPU2 is composed of 66 amino acid residues and is identical to BmK alpha2 (GenBank Accession No. AF288608) and BmK alphaTX11 (GenBank Accession No. AF155364). Double glycines are located at the C-terminal end of BmK AGP-SYPU2, which are hypothesized to be essential for its bioactivity.

However, limited availability of toxins from natural sources makes it difficult to characterize their structure-function rela-
tionships and interaction with sodium channels. In this study, we cloned the gene encoding BmK AGP-SYPU2 and successfully expressed a biologically active recombinant toxin and two mutants in E. coli in sufficient amounts, obtained by site-directed mutagenesis PCR. We focused on the C-terminal contribution to BmK AGP-SYPU2’s biological properties. Glycines at the C-terminal end (65-66) of BmK AGP-SYPU2 were deleted one by one. The first mutant was obtained by deleting one glycine at the C-terminal (Mut66) and the second mutant by deleting two glycines at the C-terminal end (Mut6566). The results of the mutagenesis, expression, purification, and analgesic activity assays of these mutants are reported here. From this study, we propose that the glycine residues are important for the analgesic activity of these mutants. Remarkably, deleting two glycine residues can alter the analgesic activity significantly.

RESULTS

Expression, purification, and characterization of BmK AGP-SYPU2 and its mutants

To evaluate the impact of the Chinese-scorpion toxin BmK AGP-SYPU2’s C-terminal end on analgesic activity, we constructed and expressed recombinant BmK AGP-SYPU2 (rBmK AGP-SYPU2), Mut66 and Mut6566 toxins using the E. coli BL21 (DE3) system. We used this system to obtain significant amounts of rBmK AGP-SYPU2 toxin and its mutated forms. The recombinant proteins were mostly expressed in soluble form, and then they were isolated from soluble fractions in the E. coli cell lysate and purified to homogeneity using IMAC (Fig. 1a, b) and cation exchange chromatography (Fig. 1c, d). The apparent molecular weight of 9 kDa was consistent with the theoretical calculated values deduced from the toxin sequence. The rBmK AGP-SYPU2 and mutants were assessed by 15% SDS-PAGE (Fig. 2a) and high performance capillary electrophoresis (HPCE) (Fig. 2b). Purity was greater than 90% as judged by Coomassie staining of the SDS-PAGE gel.

Analgesic activity assays of BmK AGP-SYPU2 and its mutants

The mouse-twisting reaction test was carried out at a dose of 2.72 \times 10^{-2} \mu\text{mol/kg} (Table 1). We studied the analgesic activity of the recombinant toxins by injecting 200 \mu l of a solution containing purified rBmK AGP-SYPU2 or its mutants intraperitoneally into mice. The analgesic activities of these sam-

Table 1. Results of twisting test of rBmK AGP-SYPU2 and its mutants

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage (\mu\text{mol/kg})</th>
<th>Twisting times (Mean ± SE)</th>
<th>Inhibition efficiency (%)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline</td>
<td>—</td>
<td>37.4 ± 2.0</td>
<td>39.40</td>
<td>100</td>
</tr>
<tr>
<td>rBmK AGP-SYPU2</td>
<td>2.72 \times 10^{-2}</td>
<td>22.7 ± 4.1*</td>
<td>39.40</td>
<td>100</td>
</tr>
<tr>
<td>Mut66</td>
<td>2.72 \times 10^{-2}</td>
<td>25.4 ± 3.1*</td>
<td>32.05</td>
<td>81</td>
</tr>
<tr>
<td>Mut6566</td>
<td>2.72 \times 10^{-2}</td>
<td>32.0 ± 3.1*</td>
<td>14.40</td>
<td>36</td>
</tr>
<tr>
<td>Morphine</td>
<td>3.51</td>
<td>20.1 ± 3.4*</td>
<td>46.26</td>
<td>117</td>
</tr>
</tbody>
</table>

*The inhibition efficiency is the ratio \((T_T)/T_0\) where \(T_0\) is the mean twisting time of the negative control group and \(T\) is the mean twisting time of the experiment groups with rBmK AGP-SYPU2, mutants and morphine. \((n=10)\). *P < 0.01 vs. rBmK AGP-SYPU2. **P < 0.01 vs. Normal Saline.
were scorpion which have high sequence identity with BmK AGP-SYPU2, sequence blast showed that the eight scorpion venom toxins, parallel αNMR (16). It exhibits the typical features of scorpion toxins: an α-helix strand on one flat face of the molecule and three anti-parallel β-sheet strands on the opposite face (Fig. 3b). Sequence blast showed that the eight scorpion venom toxins, which have high sequence identity with BmK AGP-SYPU2, were scorpion α-toxins. Therefore, we suggest that BmK AGP-SYPU2 was a scorpion α-toxin.

The modeling structures of the BmK AGP-SYPU2 mutants were obtained by DeepView (17). After molecular mechanism (MM) optimization and molecular dynamics (MD) simulation, the conformations of the two mutants with the lowest energy were chosen, and the three-dimensional structures were evaluated using the Procheck program (18). Analysis of the Ramachandran plot for Mut66 showed that 87.9% of its residues lie in the most favorable regions and 9.1% in the additional allowed regions. Analysis of the Ramachandran plot for Mut6566 showed that 84.8% of the residues lie in the most favorable regions and 15.2% lie in the additional allowed regions (data not shown).

DISCUSSION

In recent years, many efforts have been made to explore the purification, mechanism of action and the functional site of scorpion toxins using different approaches. Although a number of analogical scorpion toxins have been identified and characterized, only a few were cloned, and studies on relationships between their analgesic activity and structure are rare. Limited availability of naturally occurring toxins in the crude venom constitutes an obstacle for the biochemical and structural characterization of these proteins. Herein, we present a method for obtaining rBmk AGP-SYPU2 and its mutants in sufficient amounts to allow for structure-function studies. Difficulties exist in expressing functional small polypeptides in the cytosol of E. coli due to their degradation (19). Therefore, our strategy was to produce rBmk AGP-SYPU2 and its mutants as co-expression proteins with thioredoxin in the form of non-fusion protein (20). In this study, BmK AGP-SYPU2 and its mutant genes were cloned into a pSYPU1b vector and successfully expressed as soluble proteins that were transported into the E. coli periplasm. The pSYPU1b vector increases the solubility of products by thioredoxin. This expression system provides an easy way to purify the recombinant peptide and also allows for the production of a functional peptide without any refolding steps.

As we know, glycine is a C-terminal amide group donor for the mature toxin, which is absolutely required for peptidylglycine α-amidating monoxygenase (21). The basic residues Arg and Lys might play a role in facilitating this enzymatic amidation reaction. Most importantly, it has been reported that the C-terminal part of the scorpion toxin is involved in the sodium channel interaction (22). We found that Mut66 caused low analogical potency activity, and Mut6566 showed significantly decreased analogical potency activity (Table 1). To identify the effects resulting from structural perturbation and changes in the putative interaction with the channel receptor, homology modeling was used to examine the unmodified toxin and its mutants. The three-dimensional structures are shown in Fig. 3, and they indicate that there were changes between BmK AGP-SYPU2 and its mutants in the C-terminal region. Therefore, the C-terminal residues are probably involved in analgesic receptor interaction via conformational adjustment. We show that the C-terminal region of BmK AGP-SYPU2 plays an important role in analgesic activity and is possibly correlated with the receptor recognition site on voltage-gated sodium channels. Moreover, the alteration in analgesic activity ob-
tained with a single mutation in the C-terminal region indicated the involvement of the glycine residue in the analgesic site. Taken together, the C-terminal region, which appears to be essential for toxicity and phylogenetic selectivity, might also be important for the analgesic activity of scorpion toxins.

It is well known (23) that the orientation and strength of the dipole determine scorpion toxin activity. As shown in Fig. 3, we can see that the exposition of Site RC was reduced gradually. This could affect the exposition of the C-terminal region with the solvent and induce probable steric hindrance during interaction with the receptor. In addition, the Arg10 residue is relatively conserved among scorpion α-toxins, and this may also show the importance of Arg10’s bioactivity. The complete loss of analgesic activity of BmK AGP-SYPU2 may be due to putative interactions between Arg10 and glycines in the C-terminal region. This was deduced from careful examination of the BmK AGP-SYPU2 structure.

Our model, as well as models previously described for the insect-toxin Lqh α IT (10) and BmK M1 (an α-like toxin) (13), will allow us to more precisely define the toxin and Na+ channel contact area. It is interesting to note that BmK AGP-SYPU2 residues 65-66 are involved in analgesic activity and substitution of these two residues disturbs its biological properties. The mutagenesis results in this study add further information on not only the structure-activity relationship but also the design of genetic or synthetic mutants for future functional studies.

MATERIALS AND METHODS

Strains, materials, and animals

Bacterial strains of E. coli DH5α were used to transform the constructed vector, and the BL21 (DE3, pLys) strain was used for toxin expression using the pSYPU/1b vector (constructed in our laboratory). SP Sepharose fast flow and Chelating Sepharose Fast Flow were obtained from Pharmacia Biotech (Uppsala, Sweden). Restriction endonucleases, T4 DNA ligase and Taq DNA polymerase were obtained from TaKaRa (Dalian, China). The primers were synthesized by JINSITE (Nanjing, China). The low molecular weight marker was obtained from Shanghai Sangon Biotechnology. Kunming mice were obtained from the animal center of Shenyang Pharmaceutical University and were used in the analgesic activity assay. Other solvents and reagents were of analytical grade or better.

Site-directed mutagenesis of BmK AGP-SYPU2

Based on the amino acid sequence of BmK AGP-SYPU2, two primers were designed: an upstream T7 promoter primer (5'-tta gcc att gca tct tcc tgg ttt cat-3') and a downstream primer (5'-g gaa ttc tta acc gcc att gca tct tcc t-3'). The EcoRl restriction enzyme site is underlined for cloning the wild-type BmK AGP-SYPU2. The sequences of the mutagenic primers used to generate the desired mutations are as follows: Mut66, 5'-g gaa ttc tta acc gcc att gca tct tcc tgg ttt cat-3' (downstream primer) and Mut6566, 5'-g gaa ttc tta att gca tct tcc tgg ttt cat-3' (downstream primer). Using pET28a-AGP-SYPU2 (constructed in our laboratory) as a template, the T7 promoter primer and the three downstream primers, BmK AGP-SYPU2 and mutants Mut66 and Mut6566 were created by one-step PCR. The sample was subjected to an initial incubation at 94°C for 5 min. It was then subjected to 25 cycles for 20 s at 94°C, 30 s at 50°C, and 40 s at 72°C. The last cycle was followed by an extension step at 72°C for 8 min. The PCR products were purified and digested with Ncol and EcoRI and then ligated into the Ncol/EcoRI-digested pSYPU/1b expression vector. The resulting plasmid was transformed into BL21 (DE3, pLys) E. coli competent cells, and the positive colonies were sequenced.

Expression and purification of BmK AGP-SYPU2 and its mutants

The expression and purification of recombinant BmK AGP-SYPU2 and its mutants were primarily performed according to the Novagen pET system manual. The expression and purification procedure was performed as following: 4 liters of E. coli BL21 bacteria culture medium harboring the pSYPU-AGP-SYPU2 (mutants) expression vector was harvested by centrifugation and resuspended in lysis buffer (0.1 M Na2HPO4/NaH2PO4, 0.15 M NaCl, and 50 mM imidazole, pH 8.0). It was then sonicated on ice for 20 min. The supernatant from this sonication was collected by high-speed freeze centrifugation and applied to an IMAC column (1.0 × 6.5 cm), which was equilibrated with buffer A (0.1 M phosphate buffer saline, pH 8.0) at a flow rate of 1 ml/min. After elution with the same buffer and until a stable baseline was reached, the column was washed with buffer B (0.1 M phosphate buffer saline, pH 7.0), buffer C (0.1 M phosphate buffer saline, pH 6.0) and buffer D (0.1 M phosphate buffer saline, pH 5.0) and eluted with buffer E (0.5 M imidazole). The column was then cleaned with buffer F (0.05 M EDTA, pH 8.0). The 0.5 M imidazole eluent peak was adjusted to pH 3.6 with 0.05 M phosphoric acid. Then, the sample was applied to an SP Sepharose Fast Flow column (1.6 × 20 cm), which was equilibrated with 0.025 M citrate buffer solution (pH 3.6) at a flow rate of 1.0 ml/min. After elution with the same buffer and until a stable baseline was reached, the column was washed by gradient elution from 0 to 1.0 M NaCl in phosphate buffer saline (pH 6.6). Finally, the column was cleaned with 0.8 M NaOH. The elutropic peak containing target protein was concentrated with stirred ultra filtration cells (3 kDa membrane, Pall, NY, USA). The protein concentration was determined as described by Bradford with bovine serum albumin as a standard (24).

SDS-PAGE and HPCE analysis

Polyacrylamide gel electrophoresis (PAGE) was carried out using a 15% separation gel with a 5% stacking gel in a Tris-glycine buffer system. Samples (20 μl) were loaded onto the gel, and constant currents of 10 mA for the stacking gel and 20 mA for the separation gel were applied. The gel was stained with Coomassie brilliant blue (CBB) R-250. HPCE was performed
on a JIANGSHEN system (Dalian, China). Samples were introduced via siphon mode to an uncoated fused silica capillary (55 cm x 50 μm, inner diameter) and run in 50 mM borax buffer (pH 7.5) under 15 kV from positive to negative polarity at 20°C for 20 min. Migration of the sample was monitored by UV spectrophotometry at λ = 280 nm.

**Analogic activity assays**

The analogic activity was assessed with a mouse-twisting model as described previously (25). Adult mice weighing 18-20 g were obtained from Shenyang Pharmaceutical University. Mice were injected intraperitoneally with 0.2 ml of a 0.6% (v/v) acetic acid solution to induce extensive and long-lasting pain in their internal organs. The resulting twisting response of the mice, reflecting pain intensity, was quantified. To perform the bioassay, 0.2 ml of purified toxin solutions were injected into the mice. A 0.9% (w/v) sodium chloride solution was used as the negative control, and the pain killer morphine was used as the positive control. Twenty minutes later, 0.2 ml of a 0.6% acetic acid solution was then injected intraperitoneally. Five minutes later, the number of mouse twisting actions was counted within a 10-min period. For each sample and the controls, ten mice as a group were injected with the same dose, and the results were analyzed by a t-value test. The Institutional Animal Care and Use Committee (IACUC) approved all animal protocols.

**Sequence alignments and molecular modeling**

Sequence alignments were performed by the protein-protein BLAST (Basic Local Alignment Search Tool) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) between BmK AGP-SYPU2 and other long-chain scorpion neurotoxins. Three-dimensional structure modeling of the BmK AGP-SYPU2 mutants (Mut66 and Mut6666) was carried out by DeepView. The Amber program was used to refine the Mut66 and Mut6666 models with MM and MD. The lowest energy structures were calculated during the 1-nanosecond simulation. After removing the water molecules, the lowest energy structures were submitted for energy minimization with a conjugate gradient method for 3,000 steps. At this step, the qualities of the initial models were improved.

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