Makatoxin I, a Novel Toxin Isolated from the Venom of the Scorpion Buthus martensi Karsch, Exhibits Nitrergic Actions*

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Buthus martensi Karsch venom exhibits nitrergic action in rat anococcygeus muscle (ACM). We have purified a novel toxin, makatoxin I (MkTx I), which exhibits nitrergic action, to homogeneity from this venom by a combination of gel-filtration, cation-exchange chromatography, and reverse-phase chromatography. Its purity was assessed by capillary electrophoresis and mass spectrometry. Its molecular weight was found to be 7031.71 ± 2.88 as calculated from electrospray mass spectrometric data. The complete amino acid sequence was elucidated by sequencing of reduced and S-pyridylethylated toxin and a carboxyl-terminal peptide, F55-64, generated by the cleavage of toxin with endoproteinase Lys-C. The complete sequence of MkTx I is GRDAYIADSENCTYTCALNPYCNDLCTKNGAKSGYCQ-WAGRYNGACWCIDLPKVPISGSCR. This toxin is composed of 64 amino acid residues and contains 8 half-cystine residues. Structurally, MkTx I has high similarity to Bot I and Bot II when compared with toxins from other scorpion species. The effects of MkTx I on nitrergic responses were investigated using the rat isolated ACM mounted in Krebs solution (37 °C, 5% CO₂ in O₂). MkTx I (2 μg/ml) markedly relaxed the carbachol precontracted ACM; the relaxation was inhibited by the stereoselective inhibitor of nitric oxide synthase, N-nitro-L-arginine methyl ester (50 μM). Thus, MkTx I is the first α-toxin that can mediate nitrergic responses in the rat isolated ACM.

Scorpion venoms consist of complex mixtures of several toxins that exhibit various pharmacological activities including selective actions at sodium and potassium channels, which are the major molecular targets of scorpion toxins (1). The scorpion Buthus martensi Karsch is widely distributed in China (2), but relatively few reports have been published on the biological properties of its venom (MKV)1 or toxins. Electrophysiological studies indicate that MKV contains some neurotoxic components that possibly act on the Na⁺ channels in the excitable membrane of nerve and muscle (3), cultured mouse cardiomyocyte (4), and rat anterior pituitary cells (5).

In a preliminary study we observed that MKV produced relaxant responses of the rat precontracted anococcygeus muscle (ACM), suggesting that some constituent toxin(s) present in MKV could mimic the effects of nitrergic transmission involving the release of the inhibitory neurotransmitter nitric oxide (NO).2 Therefore, we were interested in isolating and purifying the bioactive component(s) present in the venom as well as identifying and characterizing the pure toxin(s) mediating the relaxant responses of the rat ACM. In this communication we report the isolation, purification, and complete amino acid sequence of a novel toxin, MkTx I, isolated from the venom of B. martensi Karsch and provide the first documentation of some novel nitrergic actions mediated by a scorpion toxin.

MATERIALS AND METHODS

Drugs and Chemicals—The bright white lyophilized crystals of crude venom of B. martensi Karsch were obtained from the Huazhen Pharmaceutical Animal Research Institute (Changzhou, China). Carbamylcholine chloride, Nα-nitro-L-arginine methyl ester (L-NAME), and phenolamine hydrochloride were obtained from Sigma. 2-mercaptoethanol and 4-vinylpyridine were obtained from Merck. Biogel P-2 and Bio-gel P-30 were obtained from Bio-Rad, POROS 20 HQ and POROS® R 2 H 4.6/5.0 were obtained from PerSeptive Biosystem (Cambridge, MA). Endoproteinase Lys-C was purchased from Wako Pure Chemicals (Osaka, Japan). Reagents used in the automatic protein sequencer were obtained from Applied Biosystems (Foster City, CA).

Capillary Electrophoresis—Capillary electrophoresis was performed on a BioFocus 3000 capillary electrophoresis system (Bio-Rad). The sample was injected using pressure mode (5 p.s.i/s) to a 25 μm x 24 cm coated capillary and run at 0.1 m phosphate buffer (pH 2.5) under 12.00 kV from – to – at 15 °C for 40 min. Migration was monitored at 200 nm.

Electrospray Mass Spectrographic Analysis—Samples were dissolved in deionized water and analyzed using an electrospray mass spectra system (Perkin-Elmer Sciex API III LC/MS/MS systems). The ionspray voltage was set to 4000 V. The orifice voltage was set at 75 V, and the interface temperature was set at 60 °C. Mass scan range was 500–2000 atomic mass units. Nitrogen was used as curtain gas with a flow rate of 0.6 liters/min and as nebulizer gas with a pressure setting of 30 p.s.i. A Perkin-Elmer series 200 quaternary pump was used for solvent delivery.

Protein Reduction and Pyridylethylolation—100 μg of MkTx I were dissolved in 0.5 ml of buffer solution containing 6 % guanidinium hydrochloride, 0.13 M Tris, and 0.1 mg/ml EDTA, adjusted to pH 8.0 with HCl. 1% 2-mercaptoethanol was added in 20 mol/mol disulfide bonds in the protein. The solution was then incubated at room temperature for 3 h. A solution of 10% 4-vinylpyridine was added to give a 3-fold molar ratio to 2-mercaptoethanol and incubated at room temperature for 90 min. After the reaction, the solution was loaded to a Bio-gel P-2 column (2 × 20 cm), equilibrated, and eluted with 9% formic acid for eluting. The protein peak was pooled and lyophilized.

Protein Digestion—Digestion of pyridylethylated MkTx I (100 μg)

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with endoproteinase Lys-C was performed at 37 °C overnight in 10 mM Tris-HCl, pH 8.8, containing 4 M urea (substrate:enzyme ratio was 50:1). The reaction was stopped by lowering the temperature to 4 °C. The mixtures were centrifuged, and the supernatant was fractionated by reverse-phase chromatography using a Vydac C8 column (2.1 × 150 mm) on Applied Biosystems 140B solvent delivery systems and Applied Biosystems 1000s diode array detector.

**Amino Acid Sequence Analyses**—The amino-terminal amino acid sequence of MkTx I and the sequence of the peptide P55–64 produced by endoproteinase Lys-C digestion were determined by using an Applied Biosystems 477A pulsed liquid-phase sequencer or Procise equipped with an on-line 120A phenylthiohydantoin-derivative analyzer.

**Anococcygeus Muscle Preparation**—Male Sprague Dawley rats weighing 280–360 g were stunned and exsanguinated. The paired anococcygeus muscles were dissected as described previously (6) and set up in isolated organ baths containing 7 ml of Krebs solution of the following composition: NaCl (118 mM), KCl(4.8 mM), KH2PO4 (1.2 mM), CaCl2 (2.5 mM), NaHCO3 (25 mM), MgSO4(2.4 mM), and d(+)-glucose (11 mM). The solution was maintained at 37 °C and continuously aerated with 5% carbon dioxide in oxygen. The organ responses against 1 g of tension were recorded by means of an isometric transducer (model FT 03) on a Grass polygraph. The preparation was allowed to equilibrate for about 30–40 min with changes of Krebs solution at 15-min intervals. Motor or relaxant responses of the muscle were elicited by electrical field stimulation (20–30 V, 10 Hz for 10 s, 1-ms pulse width) every 2 min.

**Nitrergic Activity**—Motor responses of the ACM to field stimulation (FS) were first blocked by phentolamine (5 µM) and the tone of the muscle was then raised by the addition of carbachol (3 µM). Relaxant nitrergic responses of the ACM to FS (15–20 min) were recorded, and FS was then stopped. Relaxant responses of the ACM to MkTx I (2 µg/ml) were then obtained in the absence of FS. Similar experiments were carried out in the presence of 50 µM L-NAME to produce blockade of the relaxant nitrergic responses of the ACM to FS. When the relaxant responses to FS were blocked, FS was stopped, and MkTx I (2 µg/ml) was then tested in the presence of L-NAME. Because the relaxant responses to MkTx I were also blocked by L-NAME, 1 µM sodium nitroprusside was then added, and the relaxant responses were recorded.

**RESULTS**

**Isolation and Purification of MkTx I**—Separation of soluble venom MKV was initially performed by gel-filtration chromatography in a Bio-gel P-30 column (2.5 × 115 cm). The lyophilized venom (500 mg) was dissolved in 10 ml of 0.05 M ammonium bicarbonate, and insoluble material was removed by centrifugation at 3500 × g for 10 min. Supernatant was loaded onto the column. Protein elution monitored at 280 nm showed 7 fractions, MK1 to MK7 (Fig. 1A). All fractions were pooled, lyophilized, and tested for nitrergic action. Only MK4 displayed nitrergic action. This low molecular weight fraction was then lyophilized, and tested for nitrergic action. Only MK4 displayed nitrergic actions, but other fractions did not display any nitrergic actions. The main fraction MK4C from cation-exchange column was dissolved in 0.02 M ammonium acetate (pH 6.0) and loaded onto a POROS® HS column (4.6 × 50 mm) equilibrated with the same buffer on a BioCAD perfusion chromatography workstation (Fig. 1B). Proteins were eluted using a linear gradient formed by mixing 0.02 M ammonium acetate (pH 6.0) and 1 M ammonium acetate (pH 6.5). Fractions were pooled according to their absorbance at 280 nm and lyophilized thricethree to remove the ammonium acetate. MK4B, MK4C, and MK4D showed nitrergic actions, but other fractions did not display any nitrergic actions.

The main fraction MK4C from cation-exchange column was dissolved in 0.1% trifluoroacetic acid and loaded onto a POROS® R 2/H column (4.6 × 50 mm), and fractions were eluted using a linear gradient of 0.1% trifluoroacetic acid with 80% acetonitrile on a BioCAD workstation, as shown in Fig. 1C, yielding three fractions, MK4C1 to MK4C3. MK4C1 did not show any nitrergic action, whereas MK4C2 and MK4C3 were found to display nitrergic activity. MK4C2 is a purified peptide and was named MkTx I. After three steps of isolation, 7.6 mg of MkTx I was purified from 500 mg of crude venom. Both capillary electrophoresis and mass spectrometry indicated that MkTx I is homogeneous (Fig. 2). The molecular weight of MkTx I determined by mass spectrometry analysis is 7031.71 ± 2.88.

**Amino Acid Sequence Determination of MkTx I**—The automatic Edman degradation of 15 pmol of S-pyridylethylated...
MkTx I proceeded with a repetitive yield of 93.7% during the first 56 cycles, which led to the unequivocal identification of the corresponding residues labeled α in Fig. 4A. From step 57 onward, the amount of identified phenylthiohydantoin-derivative was low, although the repetitive yield remained fairly good. To determine unambiguous sequence at the carboxyl-terminal end, 100 mg of S-pyridylethylated MkTx I were treated with endoproteinase Lys-C, and the resulting peptide mixtures were separated by reverse-phase high performance liquid chromatography (Fig. 3). Peptides P1–28, P33–54, and P55–64, but not P29–32, were identified by mass spectrometry analysis. All three values obtained matched the calculated molecular masses deduced from the sequences of respective peptides (Table I). The automatic Edman degradation of P55–64 allowed the identification of the carboxyl-terminal part amino acid residues. Thus, the amino acid sequence of MkTx I was established as shown in Fig. 4A, which is comparable to mass spectrometric analysis, with a calculated molecular weight of 7038.83.

The complete amino acid sequence of MkTx I was compared with known toxins from other scorpions: a striking degree of similarity was found with these toxins, for which the total primary structure is known. It is 81% similar to that of Bot II (7) and 78% similar to that of Bot I (8), both of which were isolated from North African scorpion B. occitanus tunetanus venom. From 55–77% similarity was found when compared with other toxins of Lqq IV (9) and Lqq III (10) from Leiurus quinquestriatus quinquestriatus venom, Lqhα1T (11) from L. quinquestriatus hebraeus venom, and Bom III (12) from B. occitanus mardochei venom. The least similar one was Css II

| Table I | Mass spectrometry analysis of MkTx I and peptides from Lys-C digest |  |
|---------|-------------------------------------------------|---|
| Peptide | Calculateda | Observedb |
| MkTx I  | 7038.83 | 7031.71 ± 2.88 |
| P1–28   | 3539.42 | 3538.47 ± 2.12 |
| P33–54  | 2821.81 | 2821.64 ± 1.53 |
| P55–64  | 1190.30 | 1192.99 ± 1.42 |

a Molecular masses are calculated from the amino acid sequences of these peptides.
b Molecular masses are detected by electrospray mass spectrometry.
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Nitric Oxide Synthase Inhibition by MkTx I

MkTx I is structurally homologous to other scorpion toxins so far described: (a) it consists of one single chain of 64 residues cross-linked by 4 disulfide bridges; and (b) it lacks methionine, phenylalanine, and histidine. MkTx I belongs to the α-type scorpion toxin group; it has a short J-loop (from Cys-16 to Cys-46) (14). Furthermore, it displayed a very high similarity with toxins isolated from the venoms of North American and South American scorpions. For example, Csa II showed only 25% similarity (Fig. 4B).

Nitric Actions of MkTx I—Experiments were also performed to determine whether MkTx I could mediate nitrergic responses in the rat isolated ACM because the venom (MKV) from which it was purified was observed to possess this pharmacological activity. After blockade of the adrenergic responses of the ACM by phentolamine (5 μM), FS of the carbachol-precontracted ACM produced relaxant (inhibitory) responses of the muscle. When FS was stopped, the addition of MkTx I (2 μg/ml) produced a rapid and marked 39.5 ± 3.4% (n = 4) decrease (relaxation) of the muscle tone, which then progressively returned to 82.1 ± 4.6% (n = 4) of the initial (contracted) muscle tone. Pretreatment with 50 μM L-NAME produced blockade of the nitrergic responses to FS as well as the relaxant responses to 2 μg/ml MkTx I; the relaxant responses to 1 μM sodium nitroprusside were not blocked (Fig. 5).

DISCUSSION

MkTx I is structurally homologous to other scorpion toxins so far described: (a) it consists of one single chain of 64 residues cross-linked by 4 disulfide bridges; and (b) it lacks methionine, phenylalanine, and histidine. MkTx I belongs to the α-type scorpion toxin group; it has a short J-loop (from Cys-16 to Cys-46) and a long B-loop (from Cys-36 to Cys-46) (14). Furthermore, it displayed a very high similarity with toxins of group 3 (Fig. 4B) according to the classification of Rochat et al. (15). In this group, MkTx I has highest homology with Bot II from North American scorpion B. occitanus tunetanus venom. However, it differed significantly from those of β-type neurotoxins isolated from the venoms of North American and South American scorpions. For example, Csa II showed only 25% similarity with MkTx I. Moreover, MkTx I has 5 aspartic acid and 5 asparagine residues with 1 glutamic acid and 1 glutamine residue, in contrast to β-type scorpion toxins that contain more glutamic acid and glutamine residues than aspartic acid and asparagine residues (16, 17).

The marked relaxant responses of the carbachol-precontracted ACM to MkTx I, like the nitrergic (relaxant) responses to FS, were effectively blocked by 50 μM L-NAME, a stereoselective inhibitor of the enzyme nitric oxide synthase involved in the synthesis of NO from L-arginine and oxygen (18, 19). The relaxant responses of the ACM produced by MkTx I are therefore also likely to be NO-mediated because there is now strong evidence that implicates NO as the inhibitory neurotransmitter involved in nitrergic transmission (20) and, consequently, in mediating the relaxant responses of the ACM (21, 22). Unlike the relaxant responses of the ACM to FS and MkTx I, the relaxant responses of the ACM to sodium nitroprusside, an agent that directly releases its NO via metabolism (23), were not blocked by L-NAME. This provides further evidence for the involvement of endogenous NO released prejunctionally in mediating the relaxant responses of the ACM to MkTx I.

In conclusion, a novel toxin MkTx I with nitrergic actions has been purified from the venom of the scorpion B. martensi Karsch, and the novel nitrergic actions of MKV in the rat isolated anococcygeus muscle can be attributed, at least in part, to the actions of the pure toxin MkTx I present in the venom.

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