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A K^+ channel-blocking peptide from venom of Chinese scorpion *Buthus martensii* Karsch[1]

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KEY WORDS scorpion venoms; neurotoxins; potassium channels; patch-clamp techniques

AIM: To purify and characterize a potassium channel blocker (*Bm*P-3) from the venom of Chinese scorpion *Buthus martensii* Karsch. **METHODS:** ① Purification was carried out by gel-filtration, cation-exchange, and reversed-phase chromatographies. N-terminal was directly sequenced by double-coupling manual method. Molecular weight was determined on an electrospray ionization mass spectrometer. Amino acid composition was analyzed after acidic hydrolysis for 20 h in HCl 6 mol·L^{-1} at 110 ℃. ② Toxicity tests were conducted in mice and cockroaches. ③The inhibitory effects of *Bm*P-3 on K^+ channels were tested in acutely dissociated rat hippocampal pyramidal neurons using whole-cell patch-clamp configuration. **RESULTS:** ① A pure peptide (*Bm*P-3, 8.1 mg) was obtained, about 0.08 % of total proteins of the venom. The N-terminal sequences were VGCEE and the molecular weight was 2938 in ESI-mass spectra. ② No death occurred at the dosage of 200 μg in mice and 8 μg in cockroaches. ③The peptide at 10 μmol·L^{-1} reduced the peak outward K^+ currents by 63 % ± 4 % *in vitro*. **CONCLUSION:** *Bm*P-3 inhibited K^+ channels.

The venom of scorpions has been a particularly rich source of fascinating peptides that bind to and interfere with various ion channels. For the past two decades, a large number of toxins have been isolated and characterized, and most of them acted on Na^+ channels. They were single chain polypeptides, composed of 60 – 70 amino acids and cross-linked by four disulfide bridges[1]. Recent studies showed the existence of another kind of toxins which acted on K^+ channels. They are 30 – 40 amino acid residue peptides containing three disulfide bridges[2]. The venom of Chinese scorpion *Buthus martensii* Karsch has been demonstrated to contain peptide toxins that interact with the voltage-dependent Na^+ channels of excitable cells. Furthermore, several short peptides (*Bm*P-1, *Bm*P-2, *Bm*P-3, *Bm*P-5) were also isolated from the venom. Based upon their displacement of ^{125}I-apamin from its binding sites in rat brain, *Bm*P-2, *Bm*P-3, and *Bm*P-5 were proposed to be K^+ channel blockers[2]. However, the blocking effect of K^+ channels has not been confirmed in electrophysiologic study. In the present study, we purified the toxin and studied its chemical characterization, toxicological, and electrophysiologic effects.

MATERIALS AND METHODS

Purification of the peptide Crude venom was purchased from local culture farms in Henan Province, China. It was collected by electrically stimulating the telson of scorpions. The peptide purification was carried out by several chromatographic steps starting with gel-filtration on a Sephadex G-50 column (Superfine, Pharmacia Fine Chemicals, Uppsala, Sweden). Lyophilized venom was dissolved in ammonium bicarbonate buffer 50 mmol·L^{-1} (pH 8.5) and centrifuged at 4000 × *g* for 15 min. The supernatant was applied to the column (2.5 cm × 150 cm) equilibrated and eluted with the same buffer. Subsequently, two chromatographic separations were performed on a Mono S FPLC (HR 5/5, Pharmacia LKB Biotechnology Inc) running at pH 5.0 and 4.0, respectively. The final purification was achieved on a reversed-

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phase HPLC column (C_{18}, 4.6 mm × 25 cm, 5 μm, Alltech).

Chemical characterization of the peptide Purity and molecular weight of the purified material were determined on a triple-stage quadruple mass spectrometer (Quattro VG, UK) equipped with an electrospray ionization source. Amino acid analysis was performed on a Beckman 6300 apparatus after hydrolysis of the samples in HCl 6 mol·L^{-1} at 110 ℃ for 20 h in evacuated tubes. N-terminal was directly sequenced by DABITC (4-*N*, *N*-dimethylaminoazobenzene-4'-isothiocyanate)/PITC (phenyl isothiocyanate) double-coupling manual method. DABITC and PITC were from Tokyo Kasei Kogyo Co Ltd, Japan.

Toxicity tests in mice and cockroaches The toxicity on mammals was assayed after subcutaneous injection into albino mice (Kunming strain, ♂, $n = 7$, 18 – 20 g, clean grade, Certificate № 117). Sample was supplemented with NaCl 0.15 mol·L^{-1} and 1 % bovine serum albumin (BSA, Sigma), at least 5 increasing concentrations were tested. Toxicity on cockroaches (*Blattella germanica*, 60 ± 3 mg, 2 μL for each cockroach) was monitored by injecting into the ventral intersegmental membranes. Mortality was scored 72 h after injection[3].

Electrophysiologic measurements Acutely dissociated hippocampal pyramidal neurons were obtained from 7 – 14-d-old Sprague-Dawley rats, using a modified method of protease digestion. In brief, rat was decapitated. The brain was rapidly removed and put in an ice-cold, oxygenated external solution (ES), consisting of NaCl 140, KCl 3, $MgCl_2$ 1, $CdCl_2$ 2, $CaCl_2$ 2, glucose 10, and HEPES 10 mmol·L^{-1} (pH 7.3 with NaOH). The tissue was cut into 500-μm-thick slices with a vibrator slicer (Campden 752M Vibroslice) and incubated in ES at 20 – 25 ℃ for 1 h. The slices were treated with ES containing protease XXIII (Sigma) 1 g·L^{-1} at 32 ℃ for 5 min, and placed in fresh ES containing trypsin inhibitor type II-S (Sigma) 3 g·L^{-1} for 20 min. Neurons were dissociated by triturating the tissue through a series of fire-polished Pasteur pipettes. Dissociated cells were allowed to adhere to the bottom of recording dishes, and perfused with fresh ES during the experiments. Patch pipettes (tip resistance of 3 – 4 MΩ) were pulled with Sutter P-97 puller, and filled with the pipette solution: KCl 140, HEPES 10, egtazic acid 10, $CaCl_2$ 1, $MgCl_2$ 1 mmol·L^{-1} (pH 7.4 with KOH). Membrane currents were recorded under whole-cell configuration using Axopatch 200A amplifier (Axon Instruments). The membrane potential was held at – 60 mV. Voltage pulses 200 ms long were applied from – 60 mV to + 80 mV in steps of 20 mV. Data were filtered at 3 kHz and leak-subtracted with the conditional pre-pulses, then acquired on-line by a computer through Axon DigiData-1200A interface using the pClamp 6.0 acquisition software. Tetrodotoxin (1 μmol·L^{-1}) was added in ES to abolish sodium current. *Bm*P-3 was dissolved in normal saline and applied by Rapid Solution Changer (Biologic RSC-100).

RESULTS

The soluble venom was initially separated by gel-filtration chromatography on Sephadex G-50 and gave rise to 4 fractions (Fig 1A), of which sub-fraction III was subsequently separated on a Mono S cation exchange column running in sodium acetate buffer 20 mmol·L^{-1}, pH 5.0. Five components were derived (Fig 1B). For further separation of the toxic component III-1, after dialysis and lyophilization, another Mono S column was equilibrated and run in sodium acetate buffer 20 mmol·L^{-1}, pH 4.0, and 3 fractions were afforded (Fig 1C). A pure peptide (8.1 mg) was obtained from the fraction III-1-1 on RP-HPLC column chromatography (Fig 1D) and the isolation yield was 0.08 % based on the soluble venom. In the electrospray ionization mass spectrum of the peptide, a single molecular ion peak showed homogeneity of the peptide and molecular weight of 2938 (Fig 1E). Amino acid analysis indicated that the peptide was composed of 28 amino acid residues. The amino acid sequence of N-terminal was VGCEE.

The toxicity tests of the peptide showed that no death occurred at the dosages of 200 μg/mouse and 8 μg/cockroach.

When depolarizing voltage pulses of between – 60 mV to + 80 mV were applied from a holding potential of – 60 mV, outward K^+ potassium currents were evoked. The outward

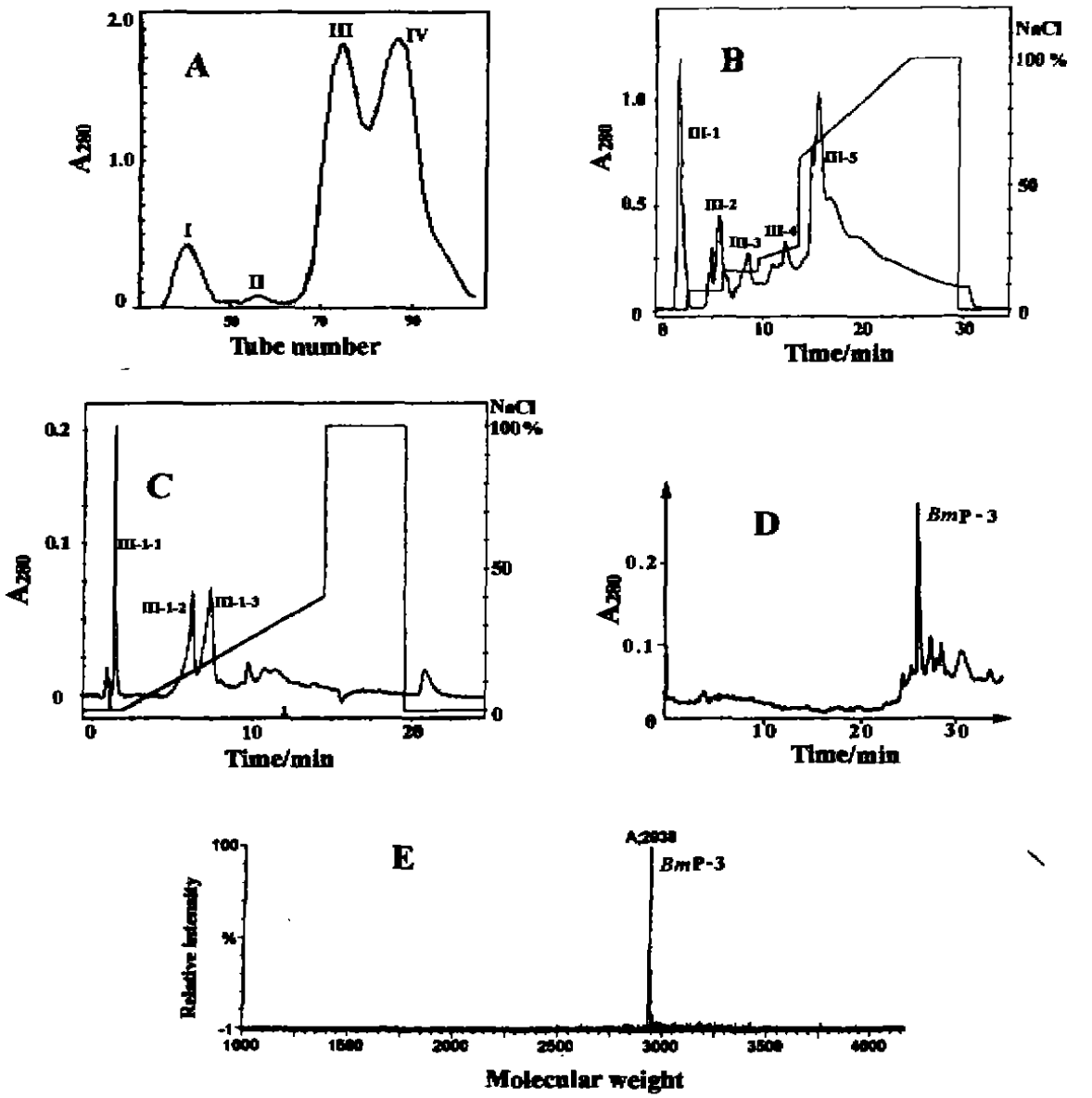

Fig 1. Purification of *Bm*P-3. (A) Gel-filtration of crude venom on a Sephadex G-50 column. (B) FPLC of fraction Ⅲ from Sephadex G-50 on a Mono S column. (C) Fraction Ⅲ-1 was reapplied to Mono S column. (D) HPLC of sub-fraction Ⅲ-1-1 on a C_{18} column. (E) Electrospray mass spectrum of *Bm*P-3.

current activated rapidly, inactivated transiently and then reached steady levels (Fig 2A). The application of *Bm*P-3 at 10 $\mu mol \cdot L^{-1}$ markedly reduced the peak currents, but did not alter the steady-state currents. Complete recovery was observed 3 min after starting wash (Fig 2A). The current-voltage (I/V) relationship of the K^+ potassium currents at peak and steady-state levels was plotted as Fig 2B. *Bm*P-3-induced inhibition of the peak currents showed a mild voltage-dependence with a reduction of 63 % ± 4 % ($n = 5$) at −20 mV.

DISCUSSION

The results derived from ESI-mass spectroscopy, amino acid analysis, and N-terminal sequence analysis clearly indicated that the peptide isolated in this study was identical to *Bm*P-3[2]. The chromatographic behavior of the peptide was also coincident with that of *Bm*P-3. Although the yield in this study (0.08 %) was similar to the previous report (0.09 %), the

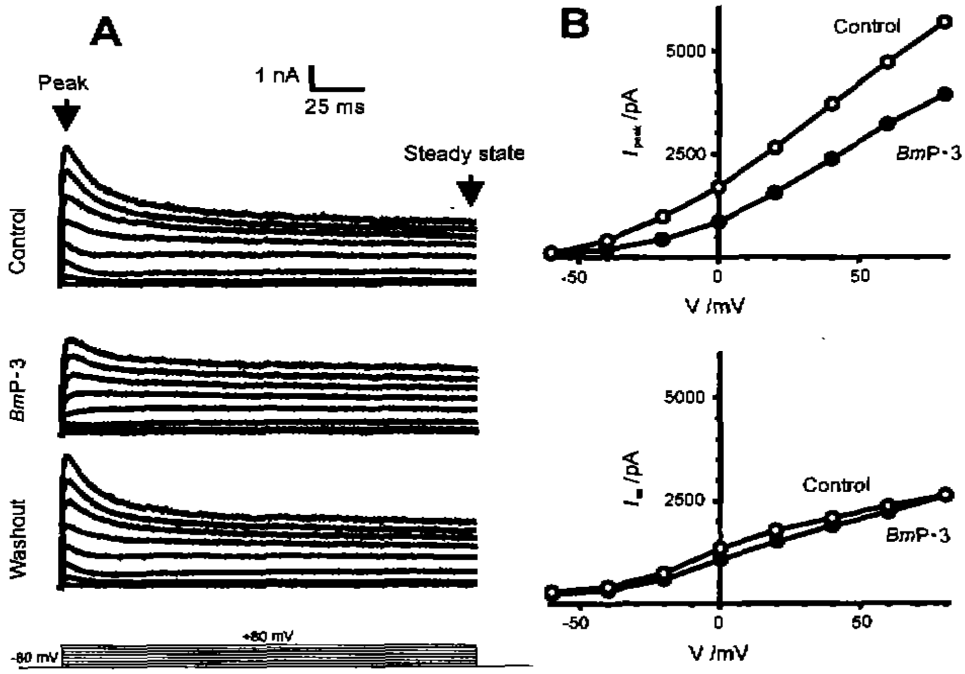

Fig 2. Effects of *Bm*P-3 on K^+ currents in hippocampal pyramidal neurons. (A) Outward K^+ currents evoked by voltage pulses of −60 mV to +80 mV. Upper: control currents. Middle: currents during application of 10 μmol·L^{-1}. Lower: currents after 3-min washing. (B) Current-voltage relationships of outward K^+ currents. Upper: I/V curves for peak currents (I_{peak}). Lower: I/V curves for steady-state currents (I_{ss}).

quantity of purified peptide is abundant (8.1 mg *vs* 91.4 μg).

*Bm*P-3 exerted few toxic effects in mice after intracerebroventricular injection of 5 μg and 10 μg[2]. The mice completely recovered in < 30 min. We found no death in mice even after subcutaneous injection of 200 μg, thus we failed to obtain its LD_{50}.

In this study we demonstrated that *Bm*P-3 inhibited the peak outward K^+ currents in hippocampal pyramidal neurons leaving the steady-state currents unchanged. In our experiments the peak outward K^+ potassium currents most likely to be I_A, and the steady-state currents I_K. Both the currents were evoked by depolarizing voltage pulses, thus mediated by activation of voltage-dependent K^+ channels. Therefore, *Bm*P-3 seems to block not only the small-conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) that are sensitive to apamin, but also voltage-dependent K^+ channels.

K^+ channel-blocking agents from scorpion venom have different primary structures, but share the same global folding: a short alpha helix and three strands of beta sheet structure, stabilized by three disulfide bridges[4]. The primary structures of *Bm*P-3 and its analogues are presented in Fig 3. It is noted that *Bm*P-3

							5					10					15					20					25				
*Bm*P-3	V	G	C	-	-	E	E	C	P	M	H	C	K	G	K	N	A	K	P	T	C	D	N	G	V	C	N	C	N	V	
Leiuropeptide I	V	G	C	-	-	E	E	C	P	M	H	C	K	G	K	N	A	N	P	T	C	D	D	G	V	C	N	C	N	V	
P01	V	S	C	-	-	E	D	C	P	E	H	C	S	T	Q	K	A	Q	A	K	C	D	N	D	K	C	V	C	E	P	I
Leiurotoxin I	A	F	C	N	L	R	M	C	Q	L	S	C	R	S	L	G	L	L	G	K	C	I	G	D	K	C	E	C	V	K	H
*Bm*P-5	A	V	C	N	L	K	R	C	Q	L	S	C	R	S	L	G	L	L	G	K	C	I	G	D	K	C	E	C	V	K	H
P-5	T	V	C	N	L	R	R	C	Q	L	S	C	R	S	L	G	L	L	G	K	C	I	G	V	K	C	E	C	V	K	H
					α									α				β				β	T	T	β				β		

Fig 3. Sequence alignment of *Bm*P-3 with other inhibitors of K^+ channels. *Bm*P-5 from *Buthus martensii* Karsch, Leiurotoxin I and leiuropeptide I from *Leiurus quinquestriatus hebraeus*, P-1 and P-5 from *Androctonus mauretanicus mauretanicus*. Secondary structure of leiurotoxin I is indicated as follow: α, α-helix; β, β-sheet and T for the turn residues.

mainly differs from *Bm*P-5, P05[5], and leiurotoxin Ⅰ[6] by missing two residues (Asn and Leu) between the first two cysteine residues and replacement of basic residues by two adjacent acidic residues (Glu4 and Glu5) in the N-terminal region. Therefore, the roles of specific amino acids in binding to K^+ channels and the relationship between the three-dimensional structure and physiological functions remain to be clarified.

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马氏钳蝎毒素中的一个钾通道阻断肽[1]

R972.
R338.7

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关键词 蝎毒；神经毒素；钾通道；膜片箝技术

目的：对马氏钳蝎毒素中的一个肽进行分离，纯化及鉴定。**方法**：①通过凝胶过滤，阳离子交换和反相色谱对样品进行纯化。用双偶合手工方法测定 N-端残基，分子量由电喷雾质谱测定。在 HCl 6 mol·L^{-1}水解后分析氨基酸的组成。②用小白鼠和蟑螂测定毒性。③在分离的海马锥体神经元上，利用全细胞记录方法测定 *Bm*P-3 对钾通道的阻断活性。**结果**：①得到 *Bm*P-3 8.1 mg，约占毒素中蛋白总量的 0.08 %。N-端序列为 VGCEE，由质谱测定分子量为 2938。②小白鼠在 200 μg，蟑螂在 8 μg 剂量下均未死。③*Bm*P-3 10 μmol·L^{-1}能使海马锥体神经元外向钾电流峰值减少 63 % ± 4 %。**结论**：*Bm*P-3 是一个钾离子通道阻断肽。