BmTx3B, a novel scorpion toxin from *Buthus martensi* Karsch, inhibits delayed rectifier potassium current in rat hippocampal neurons

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ABSTRACT

AIM: To examine the effect of BmTx3B, a novel short-chain peptide isolated from the venom of Asian scorpion *Buthus martensi* Karsch, on voltage-gated potassium channels. **METHODS:** Two types of voltage-dependent potassium currents were recorded from dissociated hippocampal neurons of neonatal rat in whole-cell voltage-clamp mode, and separated based upon their kinetic properties. **RESULTS:** BmTx3B (10-100 μmol/L) selectively inhibited the delayed rectifier potassium current (I_K), without affecting the fast transient potassium current (I_A). The inhibition of the peptide on I_K was reversible, concentration-dependent and voltage-independent. BmTx3B did not affect the steady-state activation and inactivation kinetics of the current. **CONCLUSION:** The short-chain scorpion peptide BmTx3B selectively blocked the delayed rectifier potassium channel.

INTRODUCTION

Potassium (K^+) channels play important roles in regulating various physiological functions in both excitable and non-excitable cells, such as control of action potential and excitability in nerve and muscle cells, regulation of hormone secretion, cell volume, and T lymph cell activation[1]. Molecular cloning studies have revealed enormous molecular diversity of K^+ channels: so far more than 100 pore-forming subunits of K^+ channels have been cloned. Moreover, heteromultimeric assembly of the subunits, co-assembly with auxiliary subunits, post-transcriptional processes and post-translational modifications provide a base for further diversity, thus leading to a huge number of functionally diverse K^+ channels with different biophysical, pharmacological and regulation properties[2]. Study of the structure and function of K^+ channels is dependent on the availability of specific reagents, in particular, the neurotoxins isolated from terrestrial and marine organisms. Short-chain peptides (30-40 amino acid residues) isolated from the venoms of different species of scorpions were found to specifically block distinct K^+ channels and named α-KTx toxins[3,4]. Among them, charybdotoxin (ChTX, from Israeli scorpion *Leiurus quinquestriatus hebraeus*), noxiustoxin (NTX, from American scorpion *Centruroides noxius hoffmann*) and kaliotoxin (KTX, from North Africa scorpion *Androctonus mauretanicus mauretanicus*) have been intensively studied.

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The venom of Asian scorpion *Buthus martensi* Karsch is a rich source of α-KTx toxins with 18 peptides already identified, including BmP01, BmP02, BmP03, BmP05, BmKTx, BmTx1, BmTx2, *etc*[5]. We recently isolated and purified a novel short-chain peptide BmK622 from the venom of *Buthus martensi* Karsch, and the amino acid sequence of the peptide was determined by the hybrid application of tandem CID MS/MS, Edman degradation and database search[6]. The peptide is composed of 37 amino acids including 6 cysteine residues (Fig 1), and identical with BmTx3B, a ChTX homologue found in the NCBI database (accession number AAF87224)[5]. However, thus far nothing is known about its physiological activity. In order to determine whether the peptide is a K^+ channel-blocking peptide, we investigated the effect of BmTx3B (or BmK622) on voltage-dependent K^+ currents in rat dissociated hippocampal neurons.

MATERIALS AND METHODS

BmTx3B (purity >95 %) was prepared as described elsewhere[6]. Other chemicals and reagents were purchased from Sigma, USA.

Preparation of rat dissociated hippocampal neurons Hippocampal neurons were prepared as described previously[7, 8]. Neonatal (5–9-day-old) Sprague-Dawley rats were supplied by Shanghai Laboratory Animal Center, CAS [SPF, SCXK (Shanghai) 2002-0010]. Mini-slices (500 μm) of the hippocampal CA1 region were cut in oxygenated ice-cold dissociation solution containing (in mmol/L): Na_2SO_4 82, K_2SO_4 30, $MgCl_2$ 5, *N*-[2-hydroxyethyl] piperazine-*N*'-[2-ethanesulfonic acid] (HEPES) 10 and glucose 10 at pH 7.3 with NaOH. The slices were incubated in dissociation solution containing protease XXIII 3 g/L at 32 ºC for 8 min. The solution was then replaced with dissociation solution containing trypsin inhibitor type II-S 1 g/L and bovine serum albumin 1 g/L. The slices were allowed to cool to room temperature under an oxygen atmosphere. Before recording, the slices were triturated using a series of fire-polished Pasteur pipettes with decreasing tip diameters. Dissociated neurons were placed in a recording dish and perfused with external solution containing (in mmol/L): NaCl 135, KCl 5, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10, glucose 10 and tetrodotoxin 0.001 at pH 7.3 with NaOH.

Whole-cell voltage-clamp recording The recording was made from large pyramidal-shaped neurons (10-20 μm in diameter) using an Axopatch 200A amplifier (Axon Instruments, USA) at 21-23 ºC. The electrodes (tip resistance 2~4 MΩ) were pulled from borosilicate glass pipettes (Hilgenberg, Germany). The pipette solution contained (in mmol/L): potassium gluconate 125, KCl 20, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10 and egtazic acid 10 at pH 7.3 with KOH. Voltage protocols were provided by pClamp 6.0.2 software via a DigiData-1200A interface (Axon Instruments, USA). The holding potential was -50 mV. The total K^+ currents were elicited by depolarizing command pulses from -70 mV to +60 mV in 10 mV steps following a hyperpolarizing prepulse of 400 ms to -110 mV. The delayed rectifier K^+ currents (I_K) were elicited by a similar protocol in which a 50 ms interval at -50 mV was inserted after the prepulse. Subtraction of I_K from the total K^+ current revealed the fast transient K^+ current (I_A)[9,10]. Current records were filtered at 2-10 kHz and sampled at frequencies of 10-40 kHz. Series resistance was compensated by 75 %-85 %. Linear leak and residual capacitance currents were subtracted on-line. BmTx3B was dissolved in the external solution. The toxin-containing solution was applied to the recorded neuron using RSC-100 Rapid Solution Changer with an 18-tube head (BioLogic Co France).

Activation and inactivation kinetics analysis The same pulse protocol to elicit I_K was used to inves-

	1 10 20 30	Identity
ChTX	-ZF-TNVSCTTSKECWSVCQRLHNTSRGKCMNKKCRCYS	100 %
BmTx1	-ZF-TDVKCTGSKQCWPVCKQMFGKPNGKCMNGKCRCYS	62 %
BmTx2	-ZF-TNVSCSASSQCWPVCKKLFGTYRGKCMNSKCRCYS	70 %
BmTx3A	-ZVETNVKCQG-GSCASVCRKAIGVAAGKCINGRCVCYP	43 %
BmTx3B	FGL-IDVKCFASSECWTACKKVTGSGQGKCQNNQCRCY-	40 %

Fig 1. Amino acid sequence alignment of short-chain scorpion peptides from *Buthus martensi* Karsch in the charybdotoxin subfamily: The sequences are aligned according to their cysteine residues. Gaps are presented as dashes. The percentage sequences identity with charybdotoxin (ChTX) is given at the right. BmTx1, BmTx2, BmTx3A and BmTx3B (or BmK622) from *Buthus martensi* Karsch; ChTX from *Leiurus quinquestriatus hebraeus*.

tigate its steady-state activation kinetics. Steady-state inactivation of I_K was studied by using a protocol with a series of hyperpolarizing prepulses of 600 ms from -110 to -10 mV (10 mV steps) followed by a step to a fixed voltage (0 mV). The normalized conductance is plotted against the membrane potential, and fitted with a Boltzmann equation: $G/G_{max}=1/\{1+\exp[-(V-V_H)/k]\}$, where G/G_{max} is the normalized conductance, V is the membrane potential, V_H (or V_h) is the voltage at half-maximal activation (or inactivation), and k is the slope factor.

Data analysis The amplitude of I_K was measured at a latency of 300 ms. The peak amplitude of I_A was also determined. The data are presented as mean±SD. Student's two-tailed *t*-test was used for statistical analysis.

RESULTS

With the voltage protocols and subtraction procedure used, the delayed rectifier (I_K) and fast transient K^+ current (I_A) could be simultaneously recorded from the same neuron. BmTx3B (10-100 μmol/L) selectively inhibited I_K. On contrary, the same concentration of BmTx3B did not cause a significant reduction in the amplitude of I_A (Fig 2A). The inhibition of BmTx3B on I_K was voltage-independent (Fig 2B). In the presence of 10 μmol/L BmTx3B, the amplitude of I_K in steps to 0, +20, +40, and +60 mV was reduced to 80 %±7 %, 79 %±7 %, 80 %±5 %, and 81 %±5 % of the control levels (n=6, P>0.05 *vs* 0 mV).

The inhibitory effect of BmTx3B on I_K had a rapid onset, and was reversible upon washout (Fig 3A). Furthermore, the effect was concentration-dependent. In the presence of BmTx3B 100 μmol/L, the amplitude of I_K reduced by 50 % (Fig 3B). However, the IC_{50} value was not determined due to the limitation of purified peptide obtained. The activation and inactivation kinetics of I_K were studied when BmTx3B 100 μmol/L was applied. The peptide did not result in any change in the steady-state activation and inactivation kinetics of the current (Fig 3C, 3D).

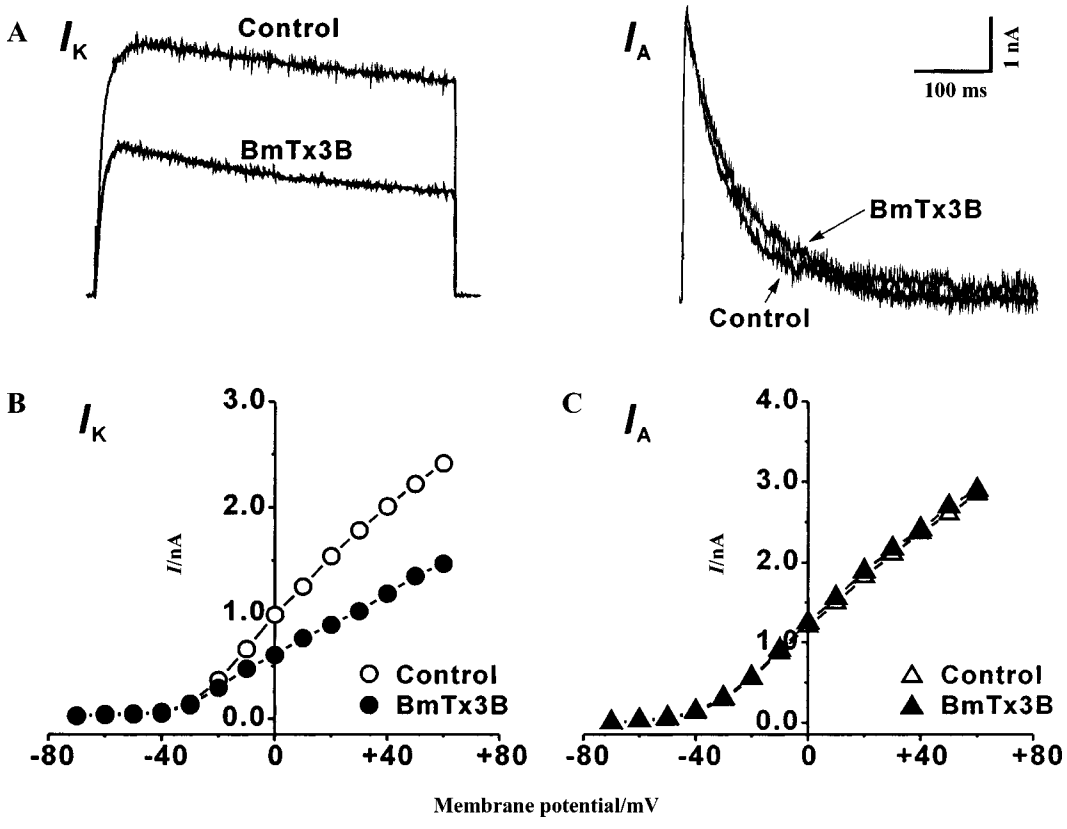

Fig 2. BmTx3B selectively inhibits the delayed rectifier K^+ current in hippocampal pyramidal neurons. (A) The left and right records are the delayed rectifier (I_K) and fast transient K^+ current (I_A), respectively, in control and in the presence of BmTx3B 100 μmol/L. (B) Current-voltage (I/V) relationship of I_K before and during application of BmTx3B 100 μmol/L. (C) Current-voltage (I/V) relationship of I_A before and during application of BmTx3B 100 μmol/L.

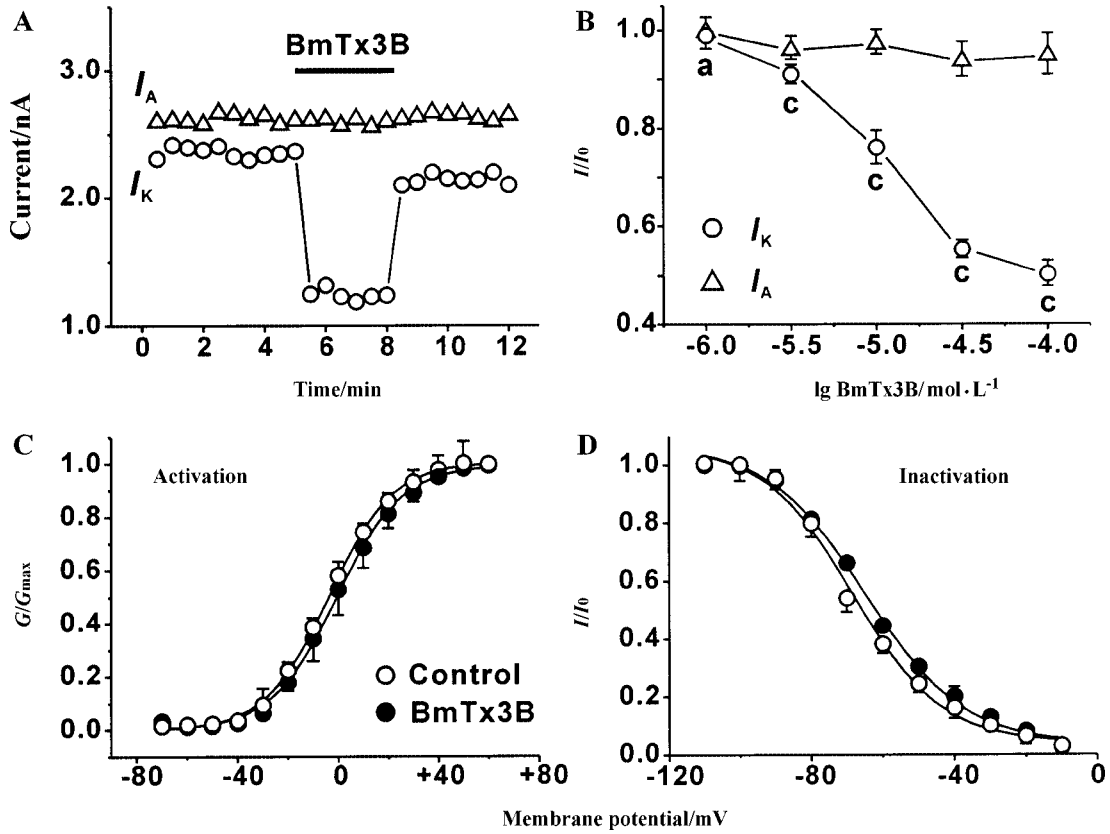

Fig 3. The inhibitory effects of BmTx3B on the delayed rectifier K^+ current. (A) Time course of inhibition caused by BmTx3B 100 mmol/L in a hippocampal neuron. (B) Concentration-response curves of BmTx3B in inhibition of I_K (n=6) and I_A (n=6). ^aP> 0.05, ^cP<0.01 *vs* the control level. (C) Steady-state activation curves (n=7), and (D) steady-state inactivation curves (n=5) of I_K constructed in control and in the presence of BmTx3B 100 μmol/L. In (C) and (D), the smooth lines show best fits with Boltzmann equations.

DISCUSSION

BmTx3B (or BmK622) shares 40 % sequence identity with ChTX (Fig 1), and has been proposed as a new member in the ChTX subfamily of α-KTx toxins (α-KTx 1.12)[5]. In the present study we demonstrated that BmTx3B exerted a selective inhibitory effect on the delayed rectifier K^+ current, without affecting the fast transient K^+ current in rat hippocampal neurons. The pharmacological profile of BmTx3B is distinct from that previously reported for other α-KTx toxins in the venom of Asian scorpion *Buthus martensi* Karsch. For example, both BmP01 and BmP02 (previously coded BmP-3) selectively inhibited the peak component of voltage-dependent K^+ current (corresponding to I_A) in rat hippocampal neurons[11,12]. BmP02 and BmP03 were also found to inhibit the transient outward K^+ current (I_{to}), without affecting the delayed rectifier (I_K) and inward rectifier K^+ current (I_{KI}) in rat ventricular myocytes[13]. In addition, BmTx3A (previously named BmTx3) was shown to be a selective inhibitor of the A-type K^+ current in rat striatal neurons in culture[14].

In addition to blocking voltage-gated K^+ channels, K^+ channel toxins in the venom of *Buthus martensi* Karsch also affected Ca^{2+}-activated K^+ channels. BmTx1 and BmTx2 were found to block rat brain Kv1.3 channels expressed in *Xenopus* oocytes, and to inhibit [^{125}I]ChTX binding to the large-conductance Ca^{2+}-activated K^+ channels (BK) on bovine aorta sarcolemmal membranes[15], whereas BmP05 and BmP03 inhibited [^{125}I]apamin binding to the small-conductance Ca^{2+}-activated K^+ channels (SK) in synaptosomal membranes of rat brain[16]. It is likely that BmKK4 mainly affects Ca^{2+}-activated K^+ channels, rather than voltage-gated K^+ channels. However, Ca^{2+}-activated K^+ current accounted for less than 2 % of the total sustained K^+ current recorded in dissociated hippocampal neurons of neonatal rat[8,17], and only a small fraction (16 %-22 %) of the current in dissociated hippocampal neurons of adult and old rat[18]. This question should be addressed using

proper methods, such as [^{125}I] ChTX binding assay[15] and [^{125}I] apamin binding assay [16].

Compared with BmTx1, BmTx2 and BmKTx, that completely block rat brain Kv1.3 channels with IC_{50} values of around 1 nmol/L[15], BmTx3B depressed I_K in hippocampal neurons with a low potency (causing a 50 % inhibition at 100 μmol/L). The deference could be explained by comparing the solution structure of BmTx3B with those of ChTX, BmTx1 and BmTx2. A mutation study has demonstrated that 5 residues (Lys^{27}, Met^{29}, Asn^{30}, Arg^{34}, and Tyr^{36}) in two C-terminal β-strands and β-turn play important roles for the binding of ChTX to Kv channel[19]. Both BmTx1 and BmTx2 have the same residues in the relevant positions, thus also exhibit high affinity for Kv channel[20]. The 4 residues (Lys^{28}, Asn^{31}, Arg^{35}, and Tyr^{37}) in BmTx3B are equivalent to the residues Lys^{27}, Asn^{30}, Arg^{34} and Tyr^{36} in ChTX. However, a different residue Gln^{30} in BmTx3B replaces the non-conservative residue Met^{29} in ChTX, which has been proposed to be crucial for its interaction with Thr^{449} in *Shaker* Kv channel[21]. It was demonstrated that mutation of Met^{29} to Gly^{29} in ChTX caused a 10^4 times increase in its K_d value towards *Shaker* Kv channel[19].

REFERENCES

1 Curran ME. Potassium ion channels and human disease: phenotypes to drug target? Curr Opin Biotech 1998; 9: 565-72.

2 Coetzee WA, Amarillo Y, Chiu J, Chow A, Lau D, McCormack T, *et al*. Molecular diversity of K^+ channels. Ann NY Acad Sci 1999; 868: 233-85.

3 Tytgat J, Chandy KG, Garcia ML, Gutman GA, Martin-Eauclaire MF, van der Walt JJ, Possani LD. A unified nomenclature for short-chain peptides insolated from scorpion venoms: α-KTx molecular subfamilies. Trends Pharmacol Sci 1999; 20: 444-7.

4 Pongs O, Legros C. Pharmacology of voltage-gated potassium channels. In: Kurachi EY, Mishina M editors. Pharmacology of ionic channel function: activators and inhibitors. Berlin: Springer-Verlag; 2000. p 177-96.

5 Goudet C, Chi CW, Tytgat J. An overview of toxins and genes from the venom of the Asian scorpion *Buthus martensi* Karsch. Toxicon 2002; 40: 1239-58.

6 Zhang NX, Wu G, Wang YF, Wang ZH, Feng CL, Wu HM. Purification and primary structure of a toxin-like peptide BmK622 from the venom of Chinese scorpion *Buthus martensii* Karsch. Acta Chim Sin 2003; 61: 630-4.

7 Sodickson DL, Bean BP. Neurotransmitter activation of inwardly rectifying potassium current in dissociated hippocampal CA3 neurons: interactions among multiple receptors. J Neurosci 1998; 18: 8153-62.

8 Li Y, Hu GY. Huperzine A, a nootropic agent, inhibits fast transient potassium current in rat dissociated hippocampal neurons. Neurosci Lett 2002; 324: 25-8.

9 Numann RE, Wadman WJ, Wong RKS. Outward currents of single hippocampal cells obtained from the adult guinea-pig. J Physiol (Lond) 1987; 393: 331-53.

10 Klee R, Ficker E, Heinemann U. Comparison of voltage-dependent potassium currents in rat pyramidal neurons acutely isolated from hippocampal regions CA1 and CA3. J Neurophysiol 1995; 74: 1982-95.

11 Wu G, Li Y, Wei D, He F, Jiang S, Hu G, Wu H. Solution structure of BmP01 from the venom of scorpion *Buthus martensii* Karsch. Biochem Biophys Res Commun 2000; 276: 1148-54.

12 Wu G, Wei DS, He FH, Hu GY, Wu HM. A K^+ channel-blocking peptide from venom of Chinese scorpion *Buthus martensii* Karsch. Acta Pharmacol Sin 1998; 19: 317-21.

13 Tong QC, Zhang Y, Li DP, Zhou ZN, Ji YH. The blocking effect of BmP02, one novel short-chain scorpion peptide on transient outward K^+ channel of adult rat ventricular myocyte. Regul Peptides 2000; 90: 85-92.

14 Vacher H, Romi-Lebrun R, Mourre C, Lebrun B, Kourrich S, Masmejean F, *et al*. A new class of scorpion toxin binding sites related to an A-type K^+ channel: pharmacological characterization and localization in rat brain. FEBS Lett 2001; 501: 31-6.

15 Romi-Lebrun R, Lebrun B, Martin-Eauclaire MF, Ishiguro M, Escoubas P, Wu FQ, *et al*. Purification, characterization, and synthesis of three novel toxins from the Chinese scorpion *Buthus martensi*, which act on K^+ channels. Biochemistry 1997; 36: 13473-82.

16 Romi-Lebrun R, Martin-Eauclaire MF, Escoubas P, Wu FQ, Lebrun B, Hisada M, *et al*. Characterization of four toxins from *Buthus martensi* scorpion venom, which act on apamine-sensitive Ca^{2+}-activated K^+ channels. Eur J Biochem 1997; 245: 457-64.

17 Santos AI, Wadman WJ, Costa PF. Sustained potassium currents in maturing CA1 hippocampal neurons. Dev Brain Res 1998; 108: 13-21.

18 Alshuaib WB, Hasan SM, Cherian SP, Mathew MV, Hasan MY, Fahim MA. Reduced potassium currents in old rat CA1 hippocampal neurons. J Neurosci Res 2001; 63: 176-84.

19 Goldstein SAN, Pheasant DJ, Miller C. The charybdotoxin receptor of a *Shaker* K^+ channel: peptide and channel residues mediating molecular recognition. Neuron 1994; 12: 1377-88.

20 Blanc E, Romi-Lebrun R, Bornet O, Nakajima T, Darbon H. Solution structure of two new toxins from the venom of the Chinese scorpion *Buthus martensi* Karsch blockers of potassium channels. Biochemistry 1998; 37: 12412-8.

21 Naranjo D, Miller C. A strongly interacting pair of residues on the contact surface of charybdotoxin and a *Shaker* K^+ channel. Neuron 1996; 16: 123-30.