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ORIGINAL RESEARCH PAPER

Functional analysis of the α-neurotoxin, BmαTX14, derived from the Chinese scorpion, *Buthus martensii* Karsch

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Abstract The gene encoding the BmαTX14 (α-neurotoxin TX14) protein, derived from the cDNA library of the Chinese scorpion *Buthus martensii* Karsch, was expressed in *Pichia pastoris.* The recombinant protein was purified by metal chelate affinity chromatography and gel filtration chromatography. Using patch-clamp technique, electrophysiological activity of rBmαTX14 was identified. In the neurons isolated from mice trigeminal root ganglion, the Na^+ current amplitude was reduced by 80% under whole cell patch-clamp recording. There were no apparent modifications to the gating mechanism in the presence of rBmαTX14. Although BmαTX14 shared a high amino acid sequence similarity with other typical α-toxins, it has different effects on neurons. Further electrophysiological analysis suggested that rBmαTX14 selectively blocked Na^+ channels and is a member of a new group of scorpion toxins.

Keywords Homology modeling · Neurotoxin · Scorpion · Sodium current channel · Trigeminal root ganglion

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Introduction

Scorpion venom is a mixture of various bioactive peptides with different functions. Accumulated data have demonstrated that scorpion neurotoxins can specifically interact with Na^+, K^+, Ca^{2+} and Cl^- channels, which changes the ion permeability of excitable cells (Possani et al. 2000; Fu et al. 2005). The scorpion toxins are powerful tools for testing the pharmacological, physiological and even structural characteristics of ion channels and their associated ionic currents (Rodriguez de la Vega et al. 2003). *Buthus martensi* Karsch (*BmK*), is a widely distributed scorpion species in Asia and has recently received increased attention. Its venom has been extensively studied and several α-toxins have been isolated (Shao et al. 1999; Peng et al. 2002). The best-studied peptides are long chain toxins containing 60–70 amino acid residues cross-linked by four disulfide bridges, which are mainly active on Na^+ channels. Toxins that bind to site 3 were called α-class. These toxins displaying α-type activity have been classified into three groups, namely: 'classic', 'anti-insect' and 'α-like' (Rodriguez de la Vega and Possani 2005).

Among studies of toxin pharmacology, the trigeminal root ganglia neurons are often used because they have the characteristic of transmitting sensory information such as touch, pressure, pain, and temperature from the peripheral region to the central nervous system (CNS). During

above signal transmitting process, Na^+ current has been widely studied for its important role (Kim and Chung 1999). In the present study, the cDNA sequence of *Bm*αTX14 has been obtained from the cDNA library of *BmK* (Zhu et al. 2000) and rBmαTX14 (α-neurotoxin TX14) protein has been successfully expressed in the *Pichia pastoris*. Using the whole cell patch-clamp recording on trigeminal root ganglia neurons, we have investigated the electrophysiological features of rBmαTX14 on the Na^+ channels of the neurons and the results suggest rBmαTX14 is a member of a new group of scorpion toxins.

Materials and methods

Materials

The cDNA library of Chinese scorpion *Buthus martensii* Karsch has been constructed by our laboratory (Zhu et al. 1999). The *Bm*αTX14 gene (GenBank ID AF156169) was screened from the cDNA library. *Pichia pastoris* strain GS115 and plasmid pPIC9K were purchased from Invitrogen (USA). T_4 DNA Ligase and restriction enzymes were obtained from TaKaRa (Japan). *Taq* DNA polymerase and dNTP were from Biostar (Canada). YNB (W/O amino acid) and peptone were made by Oxiod (UK). Metal chelate affinity chromatography column was from Invitrogen (USA). All chemicals used in the experiments were of analytical grade. Albino *Kunming* mice were purchased from Experimental Animal Center, Wuhan University.

Construction of expression vector pPIC9K-BmαTX14

PCR was used to amplify the *Bm*αTX14 coding region. To insert the amplified *Bm*αTX14 into the multi-cloning sites of pPIC9K, the forward primer (5′-GCGAATTC*CATCACCATCACCATCAC*-GTTCGGGATGCTTATA-3′) and the reverse primer (5′-CGGCGGCCGCTCAATGGCATT-TTCCTG-3′) containing *Eco*RI and *Not*I restriction sites were designed and synthesized. The underlined letters represented *Eco*RI and *Not*I restriction sites, respectively. Italic letters represented his-tag coding sequence (Lu et al. 2005). The expression vector pPIC9K-BmαTX14 was confirmed by sequencing. The positive *Pichia pastoris* GS115 strain containing pPIC9K-BmαTX14 was obtained by protocols made by Invitrogen. The expression vector pPIC9K-BmαTX14 was linearized with *Sal*I and then transformed to *Pichia pastoris* GS115. The multi-copy recombinants were screened with G418.

Expression of the recombinant BmαTX14

The positive recombinant strain *Pichia pastoris* GS115 was grown in 50 ml BMGY (buffered glycerol complex medium) at 28°C until the cells reached an OD_{600} of 4. The culture was centrifuged to collect the cells, which were suspended in 50 ml BMMY (buffered methanol complex medium). The culture was induced by 0.5% methanol for 72–84 h. The culture was then centrifuged at 10,000 *g* at 4°C for 15 min and the supernatant containing the soluble recombinant BmαTX14 was saved.

Purification of the recombinant BmαTX14

Ten milliliters supernatant was applied to metal chelate affinity chromatography (MCAC) column, washed with 100 mM imidazole buffer and the recombinant BmαTX14 was removed by eluting with 200 mM imidazole buffer. Subsequently, the fraction of the flow-through was applied to Sephadex G25 gel filtration chromatography column. The fractions of flow-through and elution peaks were collected and analyzed by 15% Tricine-SDS-PAGE.

Preparation of trigeminal root ganglion neurons

Trigeminal root ganglia neurons were prepared by the method described by Liu and Simon (1996). A pair of trigeminal ganglia were dissected from Albino *Kunming* mice (18–25 g body weight), and washed several times in Dulbecco's modified Eagle medium (DMEM)/F12 culture

solution (with 14 mM $NaHCO_3$, pH 7.4) at 4°C. After trituration with flamed Pasteur pipettes, they were incubated for 20 min at 36°C with 0.2 mg collagenase I/ml and 0.1 mg trypsin III/ml. Trypsin inhibitor was added to stop the digestion. They were triturated and washed in DMEM, and then maintained in room temperature. Cells were used in whole cell patch-clamp recording within 6 h.

Whole cell patch-clamp recording

The Na^+ current (I_{Na}) of single cell was recorded using the whole cell patch-clamp technique. The chamber was continuously perfused at a temperature of 22°C in external solution (mM): (NaCl 145, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, D-glucose 10, 4-AP 1 and TEA-Cl 20 titrated to pH 7.3 with 1 M NaOH). The solution inside the suction pipette contained (mM): CsCl 100, KF 40, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 10, HEPES 10, Na_2ATP 5 and TEA-Cl 20, titrated to pH 7.3 with 1 M CsOH. The pipette had a tip resistance of 2–5 MΩ, while the input resistance of the cells reached about 1 GΩ. The whole cell patch-clamp experiments and data acquisition were performed with a personal computer controlled EPC-10 patch-clamp amplifier operating Pulse program (HEKA Elektronik, Lambrecht, Germany). Results were shown as means ± standard error of the mean (S.E.M) of at least five experiments for each concentration. Differences between control and treatment groups were analyzed by the *t*-test. Difference at a *P*-value < 0.05 was considered to be statistically significant.

Results and discussion

Construction of the expression vector pPIC9K-BmαTX14 and the preparation of the recombinant *Pichia pastoris* strain

The 220 bp PCR product was purified and ligated into the expression vector pPIC9K to form the recombinant plasmid pPIC9K-BmαTX14. The recombinant plasmid pPIC9K-BmαTX14 was analyzed by PCR and restriction digestion (not shown). The positive clone was confirmed by DNA sequencing.

Fusion expression and purification of BmαTX14

When the supernatant of the culture after methanol induction was analyzed on 15% Tricine-SDS-PAGE, an extra protein band with an approximate molecular weight of 8.2 kDa was found (Fig. 1, lane 4). Following the purification procedure, a yield of 100–120 mg soluble rBmαTX14 per liter of culture were achieved for further analysis.

Scorpion neurotoxins have been most commonly expressed in *Escherichia coli* (Hao et al. 2005). Nonetheless, neither the quantity nor the quality of the product is satisfactory (Pang et al. 1992). In comparison with the *E. coli*, the yeast expression system can eliminate the need for refolding and correction of mispaired disulfide bonds besides the high yield of the protein product. Some other long-chain scorpion toxins have also been successfully expressed in yeast (Shao et al. 1999). Compared with the native toxin, the recombinant BmαTX14 had six additional histidine residues at the N-terminus, which seemed to have no effect on bioactivity (Deshane

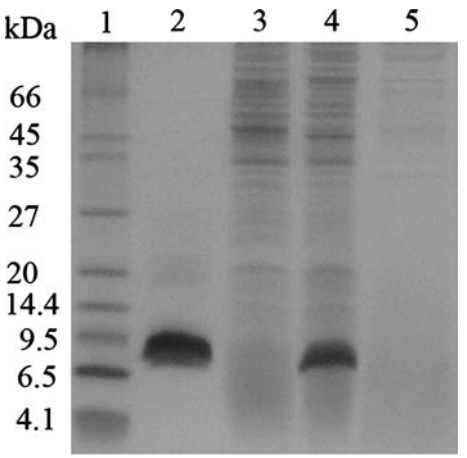

Fig. 1 Tricine-SDS-PAGE analysis of the expression and purification of rBmαTX14. Lane 1; molecular mass markers; Lane 2; purified rBmαTX14; Lane 3; uninduced fermentation supernatant of rBmαTX14; Lane 4; induced fermentation supernatant of rBmαTX14; Lane 5; induced fermentation supernatant of original plasmid

et al. 2003). Similarly, the characterization of the purified toxin demonstrates that rBmαTX14 exhibits biological activity in this work. Thus, the yeast expression system used here could be a good alternative for future small peptide expression.

Effects of rBmαTX14 on trigeminal root ganglia neuronal Na^+ channels

The effect of rBmαTX14 on the Na^+ current was tested in acutely isolated neurons from mice. In order to exclude possible effect from other kinds of currents, we tried to isolate I_{Na} from the other kinds of voltage-dependent ionic currents in whole cell patch-clamp recording by the following ways. I_K was suppressed with the use of Cs^+ in the internal solution and TEA-Cl in the external solution, and I_{Ca} was suppressed by F^- in the internal solution and Mg^{2+} in both the internal and external solutions.

The averaged and normalized current–voltage (*I–V*) relationship of the Na^+ channels in the absence and presence of 0.8 μM rBmαTX14 is shown in Fig. 2. Representative currents were evoked by step depolarization of 80 ms ranging from –60 to +70 mV, with increments of 10 mV, from a holding potential of –70 mV.

Voltage dependence of activation is shown in Fig. 3A. The curves were fitted according to the Bolzmann equation. The half-maximal potential ($V_{1/2}$) for activation and the slope factor for the voltage dependence of activation determined by the fit were –29.1 ± 1 mV and 4.1 ± 0.4 mV, in control condition, –30 ± 1.1 mV and 4.2 ± 0.6 mV in the presence of 0.8 μM rBmαTX14, respectively. The result showed that rBmαTX14 did not affect the activation (*P*-value > 0.05, *t*-test).

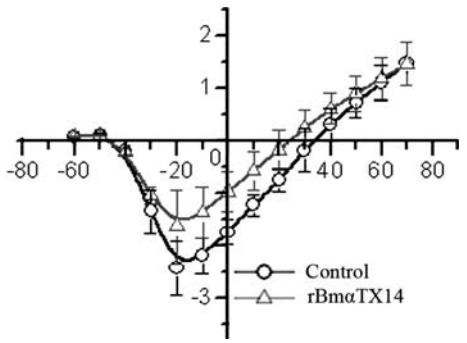

Fig. 2 Averaged and normalized *I–V* relationship in the absence (○) and in the presence of 0.8 μM rBmαTX14 (Δ)

Figure 3B displayed the voltage dependence of Na^+ channel inactivation in the absence and presence of 0.8 μM rBmαTX14. In control condition, the half-maximal potential ($V_{1/2}$) and the slope factor determined by a sigmoidal fit of the inactivation curve were –54.6 ± 1 mV and 12.7 ± 1.3 mV, respectively. In the presence of 0.8 μM rBmαTX14, $V_{1/2}$ and the slope factor were –53.2 ± 1.4 mV and 9.8 ± 1.2 mV, respectively. Clearly rBmαTX14 did not affect the inactivation too (*P*-value >0.05, *t*-test).

The Na^+ current elicited at –20 mV reached the peak amplitude under control condition. The mean current amplitude at –20 mV was approximately 2.5 nA. The traces of the Na^+ currents obtained at different concentrations of rBmα TX14 were compared with those under control condition by putting the current traces together, as shown in Fig. 4. The traces with and without rBmαTX14 were similar showing that neither the peak time nor the half-decay time was significantly modified. Thus, rBmαTX14 was proved to be a potent blocker of the Na^+ currents of trigeminal root ganglia neurons.

The effect of rBmαTX14 is concentration-dependent

The inhibitory effect of rBmαTX14 on the Na^+ channels of trigeminal root ganglia neurons was clearly concentration dependent, which was shown in Fig. 4. Currents were evoked by a step depolarization to –20 mV during 80 ms from a holding potential of –70 mV. The relative changes in the peak I_{Na} were plotted with the toxin concentration (Fig. 5). According to the relationship of the percentage decrease in I_{Na} and the toxin concentration, the EC_{50} value determined by a sigmoidal fit was 3.5 ± 0.09 μM.

The electrophysiological characterization of BmαTX14 on trigeminal root ganglia neurons is interesting. BmαTX14 shares high amino acid sequence similarity with typical α-like toxins (Fig. 6). However, BmαTX14 shows a different electrophysiological characteristic. Under whole

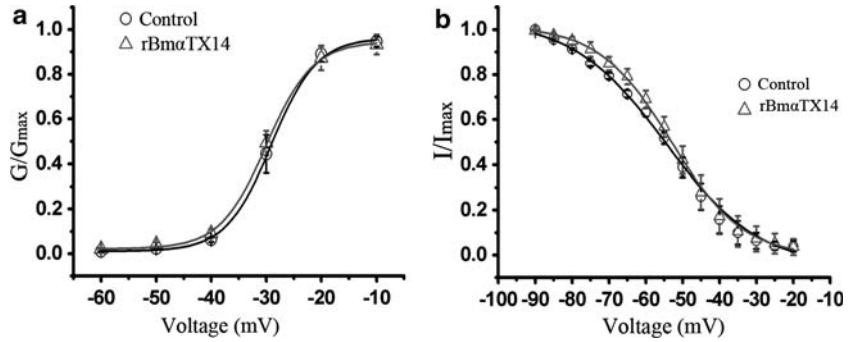

Fig. 3 The electrophysiological properties of Na^+ channels modified by rBmαTX14. (**A**) Averaged and normalized steady-state activation in the absence (○) and in the presence of 0.8 μM rBmαTX14 (Δ). (**B**) Averaged and normalized steady-state inactivation in the absence (○) and in the presence of 0.8 μM rBmαTX14 (Δ)

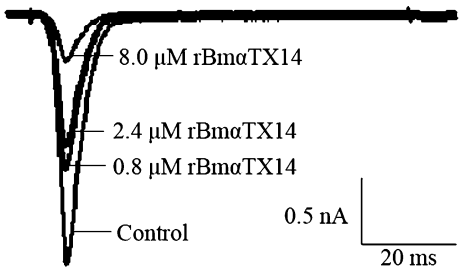

Fig. 4 Concentration dependence of the inhibitory effects by rBmαTX14 on the Na^+ channels. Current traces were evoked at –20 mV during 80 ms from a holding potential of –70 mV in the absence (control) and in the presence of increasing concentrations of rBmαTX14 (as indicated)

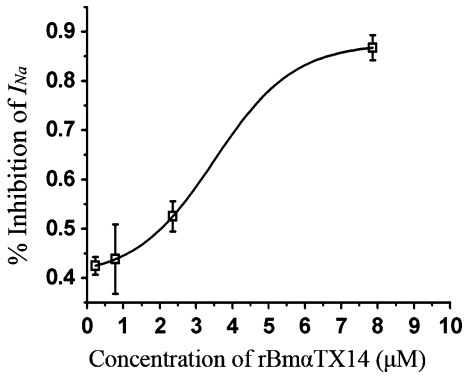

Fig. 5 Concentration–response curve of rBmαTX14 for peak Na^+ currents (I_{Na}) at a holding potential of –70 mV. The EC_{50} determined by a sigmoidal fit is 3.5 ± 0.09 μM

cell patch-clamp recording, the Na^+ current amplitude was greatly reduced in the presence of BmαTX14 while it does not modify Na^+ channel gating. This suggests BmαTX14 only performs 'blocker'-type effects on Na^+ currents of trigeminal root ganglia neurons. Indeed, the blocking effect is concentration dependent. The effect of BmαTX14 is similar to the blocking effect of Cn11, another long-chain scorpion toxin (Ramirez-Dominguez et al. 2001).

Trigeminal neuralgia is the most common form of paroxysmal facial pain. Na^+ channel blocking drugs are the first choice in treatment of trigeminal neuralgia, but clearly it is not possible for the drugs to interact with the Na^+ channels in the very same manner (Green and Selman 1991). Further studies on characteristics of the Na^+ channels are needed to identify the crucial residues of the drug-binding site. As an interesting Na^+ channel blocker of rBmαTX14, more studies on the interaction between BmαTX14 and sodium channel could provide deep clues for identifying the profile of Na^+ channel region for peptide inhibitor recognition in the near future.

Conclusions

The neurotoxin, BmαTX14 from the Chinese scorpion, has been expressed in *Pichia pastoris*. Because of the relatively protein-free property of the yeast medium and an effective secretory pathway, a simple and efficient purification protocol was developed to purify the toxin from the culture medium. This should allow large-scale production of the recombinant toxin.

Fig. 6 Comparison of the amino acid sequence of BmαTX14 and some representative scorpion α-toxins

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