Pergamon

PII: S0041-0101(96)00072-4

Toxicon, Vol. 35, No. 1, pp. 39–45, 1997

0041-0101/97 $17.00 + 0.00

BLOCKADE OF NEUROMUSCULAR TRANSMISSION BY HUWENTOXIN-I, PURIFIED FROM THE VENOM OF THE CHINESE BIRD SPIDER *SELENOCOSMIA HUWENA*

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(*Received* 29 *November* 1995; *accepted* 20 *April* 1996)

P.-A. Zhou, X.-J. Xie, M. Li, D.-M. Yang, Z.-P. Xie, X. Zong and S.-P. Liang. Blockade of neuromuscular transmission by huwentoxin-I, purified from the venom of the Chinese bird spider *Selenocosmia huwena*. *Toxicon* **35,** 39–45, 1997.—Huwentoxin-I (HWTX-I) is a neurotoxic peptide purified from the venom of the Chinese bird spider *Selenocosmia huwena*. The effects of HWTX-I on neuromuscular transmission of vertebrate skeletal muscle have been investigated by means of twitch tension and electrophysiological techniques. On isolated mouse phrenic nerve–hemidiaphragm preparations, HWTX-I blocked the twitch responses to indirect, but not to direct, muscle stimulation. The time needed for complete block of the neuromuscular transmission was dose dependent. The transmission could be mostly restored by prolonged repeated washing with Tyrode's solution. If the preparation was pretreated with D-tubocurarine and then immersed in a mixed solution of D-tubocurarine and HWTX-I, the washout time necessary to restore the neuromuscular transmission was significantly decreased. Intracellular recording at the end-plate region of frog sartorius muscle revealed that HWTX-I could synchronously reduce the amplitude of the acetylcholine potential induced by ionophoretic application of acetylcholine as well as the amplitude of the end-plate potential evoked by nerve stimulation. Both of these effects eventually disappeared; however, both could be restored by prolonged washing. Experiments on *Xenopus* embryonic myocytes indicated that HWTX-I reduced the open probability of acetylcholine-induced channel activity, and finally blocked the channel. All of these results demonstrated that HWTX-I was a peptide neurotoxin and the postsynaptic nicotinic acetylcholine receptor was its site of action. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The crude venom from the Chinese bird spider *Selenocosmia huwena* is neurotoxic to mice; it can induce paralysis of skeletal muscle and rapid respiratory failure (Liang *et al.*, 1993a).

In our previous paper, we reported that by means of reverse-phase and ion-exchange high-performance liquid chromatography (HPLC) a neurotoxic peptide, huwentoxin-I (HWTX-I), was purified from the venom of this spider. It consists of 33 amino acid residues with six Cys and has a mol. wt of 3750. Its pharmacological action, toxicity to mice and neuromuscular blockade of the isolated mouse phrenic nerve–diaphragm preparation have also been mentioned briefly (Liang *et al.*, 1993b). Furthermore, the assignment of the three disulfide bridges has been determined (Zhang and Liang, 1993). In this paper, the neuromuscular blocking action of HWTX-I and its mode of action are studied in more detail.

MATERIALS AND METHODS

Phrenic nerve–hemidiaphragm preparations

Twitch tension experiments were performed using Kunming albino mice from the animal breeding centre of Peking University. Adult mice (18–24 g of either gender) were killed by cervical dislocation. Phrenic nerve–hemidiaphragm preparations were isolated as described by Bülbring (1946) and placed in a Plexiglas chamber immersed in Tyrode's solution (composition, mM: NaCl 135.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, Na_2HPO_4 0.08, glucose 1.0, pH 7.3) or toxin solutions continuously bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at 30–32°C. The phrenic nerve was stimulated via a suction electrode at 0.2 Hz with pulses of 0.2 msec duration and supramaximal voltage. For direct stimulation, the muscle was stimulated at 0.2 Hz, 2 msec duration and supramaximal voltage. A mechanical-electric transducer made of semiconductor strain gauge was used. The signals were amplified (d.c. preamplifier Model FZG-1A, China) and recorded with a pen recorder (Model LM-6, P.R. China).

Sciatic nerve–sartorius preparations

Electrophysiological experiments were performed on isolated sciatic nerve–sartorius preparations of the frog *Rana nigromaculata*. The preparations were immersed in normal Ringer (composition, mM: NaCl 111.2, KCl 1.88, $CaCl_2$ 1.08, $NaHCO_3$ 2.38, NaH_2PO_4 0.08, glucose 11.1, pH 7.4) or modified low Ca^{2+} Ringer solution (composition, mM: $CaCl_2$ 0.3–0.5, $MgCl_2$ 1.5, otherwise the same as normal Ringer; McManus and Musick, 1985) to reduce the amplitude of end-plate potentials (EPP) and to block neuromuscular transmission. Conventional intracellular recording techniques were used. Glass microelectrodes filled with 4 M potassium acetate (KAc), and resistances ranging from 10–15 MΩ, were used in all experiments. The Ag–AgCl wire from the microelectrode was connected to the input of a preamplifier (Model MEZ-8201, Nikon, Japan). The output from the preamplifier was displayed on an oscilloscope (Tektronix 5113, U.S.A.), and photographed. The Ag–AgCl reference electrode was inserted in the bath solution. The responses to ionophoretically applied acetylcholine (ACh) and the EPP induced by nerve stimulation were recorded from end-plate regions of surface muscle fibres. For ionophoretic application of ACh (Del Castillo and Katz, 1955), the Microiontophoresis Current Programmer (Model 260, WPI, U.S.A.) was used. The positive ejected current pulses were adjusted to 150 nA and 20 msec duration controlled by a stimulator. Retaining currents of 10 nA were used. All experiments were carried out at 22–25°C.

Recordings of single acetylcholine receptor channel currents on Xenopus *embryonic myocytes*

Cell culture. The embryonic myocytes were prepared as described by Spitzer and Lamborghin (1976) and Xie *et al.* (1989). Briefly, when fertilized eggs reached stage 20–22 (Nieuwkoop and Faber, 1967), their coats were removed. Posterior neural plates and a small amount of underlying mesoderm adhered to neural plates were dissected and placed in Ca^{2+}–Mg^{2+}-free Ringer supplemented with EDTA (composition, mM: NaCl 67, KCl 1.6, HEPES 8, EDTA 1, pH 7.8). The cells were plated on clean glass coverslips and used for experiments after incubation for 20–30 hr at 23°C. The culture medium consisted of 30% (v/v) L-15 Leibovitz medium (Gibco, U.S.A.), 2% (v/v) fetal bovine serum (Gibco, U.S.A.), 68% (v/v) Ringer (composition, mM: NaCl 115, $CaCl_2$ 2, KCl 2.5, HEPES 10, pH 7.4).

Single channel recording. Isolated spherical myocytes were used in the experiments. Single AChR channel currents were recorded using cell-attached patch-clamp configuration by a patch-clamp amplifier (List EPC-7, Germany) and displayed on an oscilloscope. Recordings were made at room temperature (20–23°C) in culture medium as the bath solution. The recording pipettes were filled with Ringer in which 0.5 μM ACh was added, sufficient to produce significant channel activity without desensitization of the AChRs. The recording currents were filtered at 3 kHz and stored on a tape recorder (TEAC XR 30C, Japan). Records were replayed from tape,

and data were acquired via Labmaster TL-1 (Axon Ins., U.S.A.), and analysed on a PC 386 computer using pCLAMP version 5.5.1 (Axon Ins.).

Huwentoxin-I was prepared as described in the previous paper (Liang *et al.*, 1993b). Stock solutions of HWTX-I (1×10^{-2} g/ml in redistilled water) were stored at 4°C and diluted in the Tyrode's or Ringer solution on the day of experiment. D-Tubocurarine, HEPES, EDTA and AChCl were purchased from Sigma.

RESULTS

Effects of huwentoxin-I on mouse phrenic nerve–diaphragm twitch tension

In control experiments with the preparations immersed in Tyrode's solution, there was no significant change in the twitch responses within 4 hr. HWTX-I inhibited the nerve-evoked twitch tension. In all experiments after a latent period of varying length, depending on the concentration of toxin, the twitch responses began to decline gradually, without any initial augmentation, fasciculation or contracture; finally the muscle was completely paralysed. After blockade, the direct stimulation was unaffected, although the responses were usually decreased to 70–80% of control. In the previous paper (Liang *et al.*, 1993b) we reported that the neuromuscular block was irreversible, since there was no sign of recovery after 30 min washing with Tyrode's solution. However, with prolonged repeated washing for more than 40 min, first signs of responses to nerve stimulation began to appear, and recovery to 60–80% of control could occur after another 40 min of washing. An example is shown in Fig. 1, exhibiting the effect of 3×10^{-6} g/ml HWTX-I on twitch height to both indirect and direct stimulation. The time from the application of toxin to complete block of neuromuscular transmission was defined as blocking time, and was dose dependent. The four concentrations of HWTX-I we used were (in g/ml) 1×10^{-5}, 3×10^{-6}, 1×10^{-6} and 5.5×10^{-7}, and the blocking times were (mean $\pm$ S.D. in minutes) 15.2 ± 1.9 ($n = 5$), 24.2 ± 2.4 ($n = 9$), 81.8 ± 9.4 ($n = 5$) and 189.8 ± 10.9 ($n = 5$), respectively.

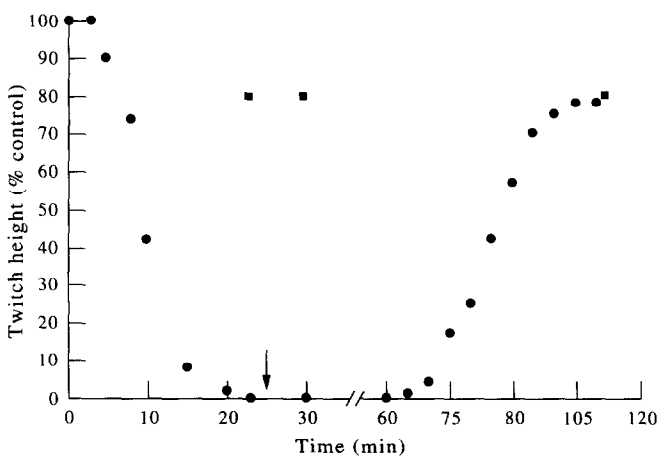

Fig. 1. Effects of 3×10^{-6} g/ml HWTX-I on the twitch responses of mouse phrenic nerve–hemidiaphragm muscle.

Nerve-evoked (●) and directly elicited (■) muscle twitch responses, expressed as percentage of control twitch height (100%), are plotted against time in minutes after toxin application at time 0. The transmission was blocked at 23 min. At 25 min (arrow) washing with Tyrode's solution was begun, and at 65 min the first sign of restoration to nerve-evoked responses began to appear.

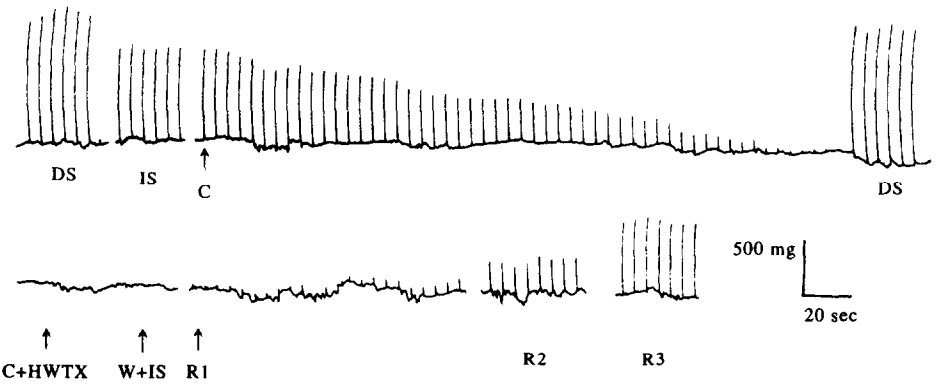

Fig. 2. Effect of pretreatment with D-tubocurarine before the addition of HWTX-I on the recovery process during washout period.
DS, Direct stimulation; IS, indirect stimulation; C, 1×10^{-5} g/ml D-tubocurarine was applied, 4.5 min later neuromuscular transmission was blocked; C + HWTX, the preparation was incubated in the mixed solution of D-tubocurarine and HWTX-I for 30 min; W + IS, repeated washing with Tyrode's solution and indirect stimuli were given; R1, 9 min after washing, responses to nerve stimulation appeared; R2, responses at 15 min after washing; R3, responses at 35 min after washing.

Effect of pretreatment with D-*tubocurarine on the blocking action of huwentoxin-I*

To test the effect of treatment with D-tubocurarine prior to the addition of toxin on the blocking action of HWTX-I, the experimental procedures used by Chiappinelli and Zigmond (1978) were followed.

With 1×10^{-5} g/ml D-tubocurarine alone, the single twitch responses of the diaphragm preparation elicited by nerve stimulation were depressed immediately, and neuromuscular transmission was blocked in 4.5 ± 0.8 min (mean $\pm$ S.D., $n = 5$). After blockade, followed by washing with Tyrode's solution, the first sign of recovery from block appeared in 1–2 min, and within 10 min the twitch responses were fully recovered (results not shown). If the preparation was first immersed in 1×10^{-5} g/ml D-tubocurarine solution, and after blockade a mixture of D-tubocurarine plus HWTX-I solution was applied, the final concentrations were 1×10^{-5} g/ml and 3×10^{-6} g/ml, respectively. The preparation was incubated in the mixed solution for 30 min, then washed with D-tubocurarine and toxin-free Tyrode's solution. In seven preparations, the first sign of recovery to nerve stimulation appeared between 8 and 12 min, and recovery to 80–90% of control took place within 35 min of washing (Fig. 2).

Effects of huwentoxin-I on the acetylcholine potential elicited by ionophoretically applied acetylcholine and end-plate potential evoked by nerve stimulation

Frog nerve sartorius preparations were bathed in modified low Ca^{2+} Ringer solution. Only preparations giving constant EPP to nerve stimulation and stable ACh potential to ionophoretic delivery of the same quantity of ACh during an initial 30 min period were used as controls. The bathing solution was changed to 1×10^{-5} g/ml HWTX-I, and both ACh potential and EPP were gradually and synchronously depressed, and finally disappeared. Both could be restored by repeated washings (Fig. 3). The time needed for complete abolition of the potentials was 35.4 ± 1.8 min for EPP and 34.4 ± 1.5 min for ACh potential (mean $\pm$ S.D., $n = 5$).

Effects of huwentoxin-I on the activity of acetylcholine receptor channel

The recording pipette was first made with a quick dip into 0.5 μM ACh Ringer to fill the tip with ACh alone without the toxin, and the shaft of the pipette was filled with 0.5 μM ACh plus 3×10^{-5} g/ml HWTX-I Ringer. Under this condition, after rapid sealing of the pipette in the first minute the channel activity in the presence of ACh alone could be recorded as a control. When the toxin diffused to the tip, the channel activity of the same patch in the presence of toxin was recorded and could be compared with the control.

In all experiments the pipette potential was clamped at + 20 mV. An experiment illustrating the effect of 3×10^{-5} g/ml HWTX-I on the activity of the AChR channel is shown in Fig. 4. The channel-open probability was 24.0% within 1 min after sealing, but it was significantly reduced by the toxin. At 5 min after sealing, the channel-open probability was 3.8%, more than 80% lower than that of the control. The channel-open time and current amplitude were also decreased, from 2.8 msec and 2.7 pA (control) to 1.8 msec and 2.3 pA (at 5 min), respectively. Finally, the channel was blocked at 12 min after sealing (11.5 $\pm$ 0.5 min, mean $\pm$ S.D., $n = 5$).

DISCUSSION

Using pharmacological and electrophysiological methods, we found that HWTX-I blocked the nerve-evoked twitch tension of mouse diaphragm. After blockade, the responses to direct muscle stimulation remained unaffected, although the twitch tension was a little depressed in most preparations. The blockade was slowly reversible: after a washout period of more than 40 min, responses to nerve stimulation began to appear. If the preparation was first treated with D-tubocurarine, after blockade, then incubated for 30 min in a mixture of D-tubocurarine and HWTX-I, 30 min was long enough for

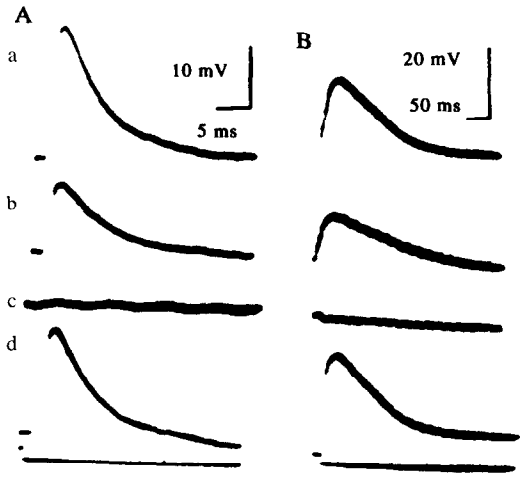

Fig. 3. Effect of 1×10^{-5} g/ml HWTX-I on responses to nerve stimulation (EPP, column A) and ionophoretic application of ACh (ACh potential, column B) recorded from frog nerve sartorius preparation in low Ca^{2+} Ringer.
(a) Control, before addition of toxin; (b) 20 min after addition of toxin; (c) 36 min after addition of toxin, the responses were eliminated; (d) 38 min after washout. The bottom lines are the stimulus marker and ionophoretic pulse, respectively.

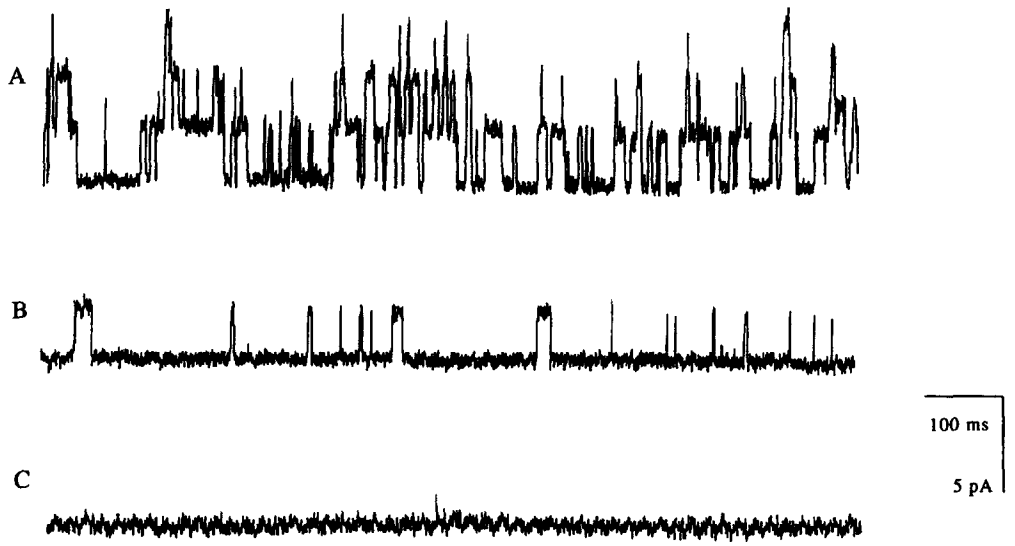

Fig. 4. Effect of 3×10^{-5} g/ml HWTX-I on ACh-induced channel activity recorded on cultured *Xenopus* embryonic myocyte.
(A) Control, immediately after sealing with 0.5 μM ACh in the tip of pipette; (B) 5 min later; (C) 12 min after sealing the channel, activity was blocked (pipette potential + 20 mV).

HWTX-I alone at the concentration used to block neuromuscular transmission. However, during the subsequent washout period, the time taken to restore transmission was significantly decreased. This result suggested the possibility of D-tubocurarine and HWTX-I interacting with the same target site. Since the transmission was blocked by pretreatment with D-tubocurarine, and the majority of the postsynaptic receptors were already combined with D-tubocurarine, it is reasonable to assume that during the incubation period only a small number of receptors could combine with HWTX-I; therefore, the washout period significantly decreased. Chiappinelli and Zigmond (1978) investigated the effect of α-bungarotoxin on the synaptic transmission in the avian ciliary ganglion. They found that incubation of ganglia with D-tubocurarine prior to the addition of α-bungarotoxin significantly decreased the duration of the washout period necessary to restore transmission. They concluded that D-tubocurarine and α-bungarotoxin are interacting with the same receptor. Here, we obtained the same result. If the target site of HWTX-I was other than the receptor, then the recovery time should be at least as long as HWTX-I acting alone. Experiments on ionophoretic application of ACh demonstrated that the toxin reduced the amplitude of the ACh potential as rapidly as it reduced that of EPP, and HWTX-I could block the activity of AChR channel of *Xenopus* embryonic myocyte, providing further evidence to support the assumption that the blocking action of HWTX-I was postsynaptic and nAChR was its site of action, as with α-snake toxins and α-conotoxins from marine snails (Adams and Swanson, 1994). However, the primary structure of HWTX-I showed no homology with the sequences of α-snake toxins (Lee, 1979) and α-conotoxins (Gray *et al.*, 1981; Nishiuchi and Sakakibara, 1982). Furthermore, we have noted that the pattern of disulfide linkage between HWTX-I and ω-conotoxin GVIA from the marine cone snail (Pallaghy *et al.*, 1993) is similar; ω-conotoxin is a Ca^{2+} channel blocker, having an entirely different function

from HWTX-I. Therefore further investigation into the structure–function relationship between HWTX-I and ω-conotoxin GVIA would be interesting.

Acknowledgements—We are grateful to Prof. Xiao-Cheng Gu for her valuable comments on the manuscript. This work is supported by National Joint Laboratory of Biomembrane and Membrane Biotechnology, Chinese Academy of Sciences (to P.-A. Zhou) and National Natural Science Foundation of China (to S.-P. Liang).

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