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The effect of Huwentoxin-I on Ca^{2+} channels in differentiated NG108-15 cells, a patch-clamp study

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Abstract

Huwentoxin-I (HWTX-I), a 3.75 kDa peptide toxin isolated from the venom of the spider *Selenocosmia huwena*, was found to be a reversible presynaptic inhibitor by our previous work. Using whole-cell patch clamp methods, we found that HWTX-I had no significant effect on the TTX-sensitive Na^+ current or the delayed rectifier K^+ current (K_r) in low-serum medium cultured NG108-15 cells, but High-Voltage-Activated Ca^{2+} channel expressed in prostaglandin E_1 differentiated NG108-15 cells could be potently inhibited by HWTX-I ($EC_{50} \approx 100$ nM), while it hardly affected low-voltage-activated Ca^{2+} channel. Among types of high-voltage-activated Ca^{2+} channel, HWTX-I selectively inhibited N-type Ca^{2+} channel and had only very weak effect on L-type Ca^{2+} channel in prostaglandin E_1 differentiated NG108-15 cells.

Keywords: *Selenocosmia huwena*; Neurotoxin; Presynaptic inhibitor; Voltage-gated Ca^{2+} channels; NG108-15 cell; Whole-cell patch clamp

1. Introduction

It is widely accepted that Ca^{2+} influx is a key factor in neurotransmitter release from presynaptic nerve terminals (Harald, 1983). Voltage-gated Ca^{2+} channels are the targets of many toxins such as, ω-Conotoxins (Mccleskey et al., 1987), ω-Agatoxins (Mintz et al., 1991) and Funnel-web spider toxins (Lin et al., 1990). Generally, voltage-gated Ca^{2+} channels can be classified as low-voltage-activated Ca^{2+} channel (LVA-Ca^{2+} channel, or T-type Ca^{2+} channel) and high-voltage-activated Ca^{2+} channel (HVA-Ca^{2+} channel). HVA-Ca^{2+} channel consists of L-type, N-type and P/Q-type Ca^{2+} channels (Miller, 1987, 1992; Nowycky et al., 1985). Among multiple types of voltage-gated Ca^{2+} channels, N-type and P/Q-type Ca^{2+} channels seem to be the most critical for modulating neurotransmitter release (Hirning et al., 1988;Waterman, 1997).

HWTX-I, a neurotoxin isolated from the venom of the Chinese spider *Selenocosmia huwena*, can reversibly block the neuromuscular transmission in an isolated mouse phrenic nerve-diaphragm preparation (Liang et al., 1993). The amino acid sequence has been determined as the following: NH_2–A–C–K–G–V–F–D–A–C–T–P–G–K–N–E–C–C–P–N–R–V–C–S–D–K–H–K–W–C–K–W–K–L–COOH (Liang et al., 1993). The three-dimensional structure in aqueous solution has been determined by 2D-NMR (Qu et al., 1997). The successful chemical synthesis of HWTX-I has also been achieved (Liang et al., 1997). Zhou et al., (1997) suggested that HWTX-I blocks postsynaptic ACh

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Table 1
Compositions of solutions (the first column gives symbols that are used in the text to indicate the respective solution)

External solution	$BaCl_2$ (mM)	$CdCl_2$ (mM)	NaCl (mM)	KCl (mM)	$MgCl_2$ (mM)	Glucose (mM)	Na-Hepes (mM)	TTX (μM)	pH
Ba	10		145	5.5	1	10	10	0.1	7.2
Cd		2	145	5.5	1	10	10		7.4

Internal solution	KCl (mM)	CsCI (mM)	$MgCI_2$ (mM)	ATP (mM)	MgATP (mM)	Phosphocreatine Di-Tris salt (mM)	EGTA (mM)	Na-Hepes (mM)	pH
Ca		145			1		10	10	7.2
Na–K	145		1	3		5		10	7.4

receptors based on the result that D-tubocararine and HWTX-I showed competitive effects in the blockade of neuromuscular transmission. However, experiments in our laboratory on the binding of the radioisotope labeled HWTX-I to the Torpedo electric organ gave completely negative results. We also have observed no effect of HWTX-I on the nicotinic ACh receptor in the TE671 human muscle cell line. These results are suggestive of the complicated nature of the physiological activity of HWTX-I. Through three different kinds of isolated nerve-synapse preparations: guinea pig ileum, rat vas deferens and toad heart, HWTX-I was determined to block neuromuscular transmission in each case, but it did not affect the postsynaptic effect of acetylcholine or norepinephrine (Liang et al., 2000). So, we concluded that HWTX-I might inhibit the release of neurotransmitter from the nerve terminal of both, the cholinergic and the adrenergic synapse.

In order to investigate the exact site of the presynaptic action of HWTX-I, we conduct experiments of its influence on TTX-sensitive Na^+ current, delayed rectifier K^+ current (K_r) and Ca^{2+} currents in NG108-15 cells using whole-cell patch clamp configuration. Here, we report the results.

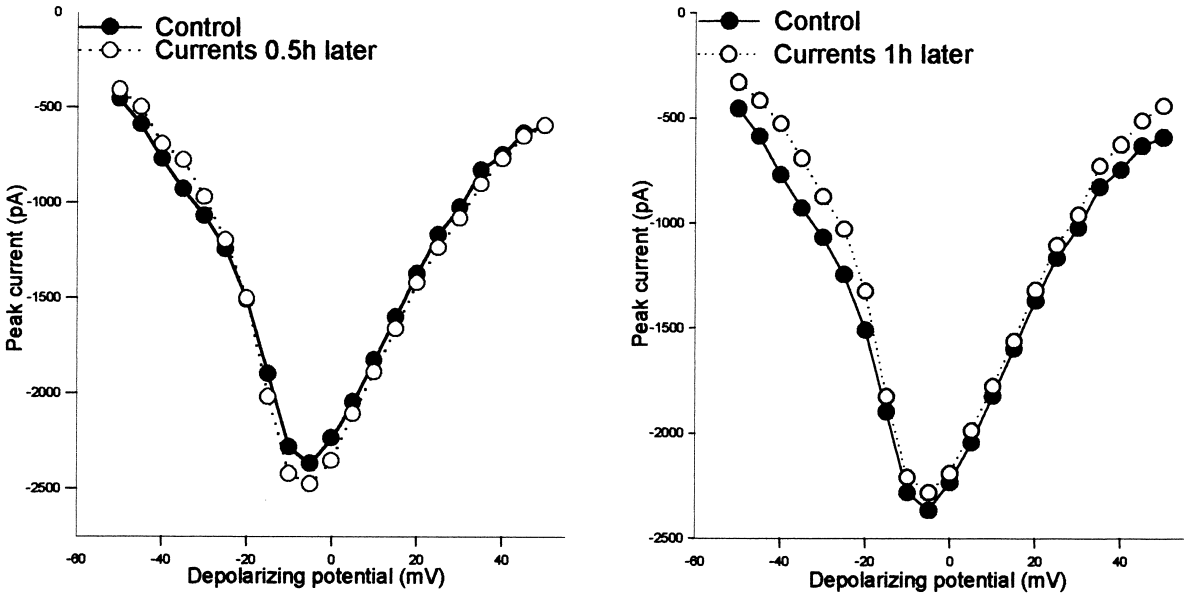

Fig. 1. Rundown of Ba^{2+} currents ($n = 3$). The left: I–V relationship comparison of Ba^{2+} currents obtained half an hour later with control; the right: I–V relationship comparison of currents obtained 1 h later with control. Each point represented peak amplitude of currents elicited by 80 ms voltage steps to respective membrane potentials ranging from −50 to +50 mv with a ΔV of 5 mV from a V_h of −90 mV. (Cell 322).

2. Materials and methods

2.1. Toxin purification

HWTX-I was purified using reverse phase HPLC followed by ion-exchange chromatography as described earlier (Liang et al., 1993).

2.2. Tissue culture

NG108-15 cells (from Dr. Gang Pei, Shanghai Cell Institute) were stored in −70°C refrigerator instead of liquid nitrogen, and retrieved into culture flasks every month. The cultured medium was composed of 90% Dulbecco's modified Eagle's medium, 10% newborn calf serum, hypoxanthine aminopterin thymidine supplement and penicillin-streptomycin (adapted from Kasai and Neher, 1992). Another 5%-serum medium with similar composition as that was used to record sodium and potassium currents. Three to five days prior to recording calcium currents, cells were transferred into 35 mm culture dishes (Corning, Sigma). Electrophysiological experiments were made with these plates; the cells in these plates were supplied with a low-serum medium composed of 1% calf serum, 98% DMEM, HAT and antibiotics, with the addition of prostaglandin E_1 (10 μM) and 3-isobutyl-1-methylxanthine (50 μM) (adapted from Kasai et al., 1992; Lukyanetz, 1998).

2.3. Solutions and drugs

The composition of solutions used in this study is listed in Table 1. The whole-cell patch clamp was performed in Ba external solution and patch pipettes were filled with Ca internal solution, unless otherwise stated. For recording sodium and potassium currents, the cells were bathed in Cd external solution and patch pipettes were filled with Na–K internal solution. Each solution was membrane (0.22 μm) filtered and internal solutions were stored at −20°C. All experiments were performed at room temperature (20–25°C).

To make stock solution, nifedipine (Sigma, St. Louis., USA) was dissolved in DMSO at 5 mM, synthetic ω-conotoxin GVIA (Advanced Chemtech, Louisville, Kentucky, USA) was dissolved in distilled water at 300 μm, purified HWTX-1 was dissolved in distilled water at 200 μM. These drugs were stored at −20°C and were diluted into the external solution before use.

2.4. Whole-cell recording

Macroscopic Ca^{2+} channel currents (filtered at 10 kHz, digitized at 3 kHz with a EPC-9 patch clamp amplifier, HEKA Electronics, Germany) were recorded at room temperature (20–25°C) in differentiated NG108-15 cells, using Ba^{2+} as the charge carrier. An agar salt bridge was introduced between bath electrode and bathing solution to avoid disturbing the composition of the external solution. Spherical and elliptic cells (about 100–200 pF size) without prominent long dendrites are favourable for recording, for large and stable currents were more frequently available from these cells. Borosilicate glass pipettes (2–3 μm tip diameter, 1–2 MΩ resistance) were obtained with a two sequential pulling from a micropipette puller (PC-10, Narishige, Olympus), and cells were clamped with these pipettes. Drug-containing solutions of about 10 μl volume were applied by pressure injection with a microinjector (IM-5B, narishige, Olympus) through a micropipette (30–50 μm tip diameter) placed at a distance of 80–100 μm from the cell. Linear leak current was subtracted by using current from hyperpolarizing pulses; access resistance was kept in the range of 5–10

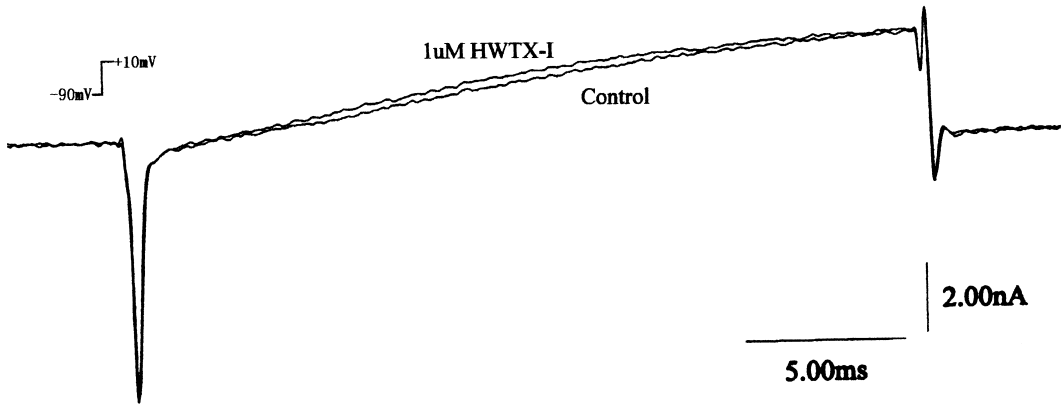

Fig. 2. HWTX-I did not affect Na^+ or K^+ currents ($n = 3$). The TTX-sensitive Na^+ current and the delayed rectifier K^+ current K_r were elicited by 25 ms voltage steps to +10 mV from a V_h of −90 mV, currents were almost unaffected by 1 μM HWTX-I ($n = 3$). (Cell 809).

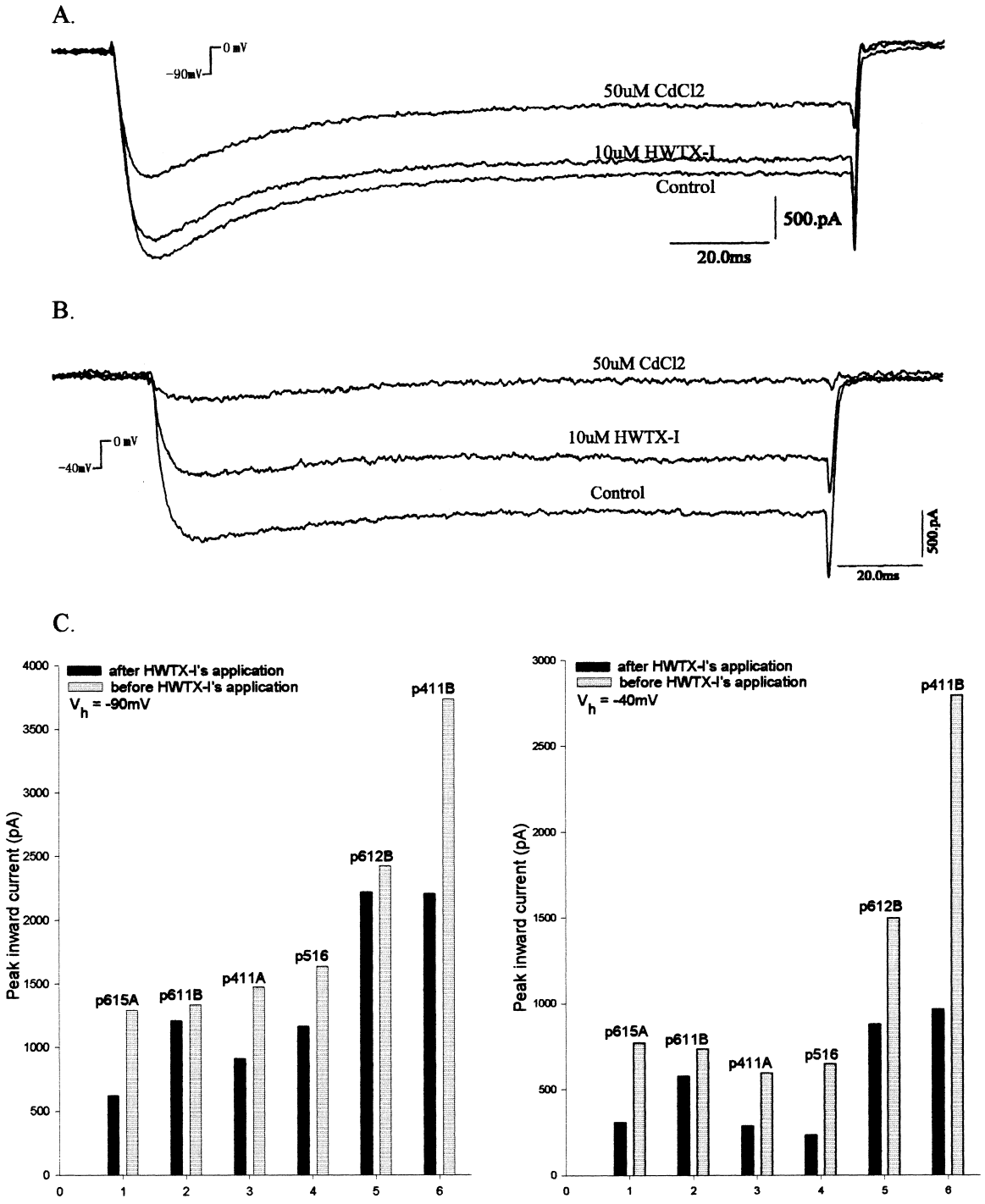

Fig. 3. HWTX-I's effects on the whole Ba^{2+} currents and the HVA Ba^{2+} currents. (A) HWTX-I blocked a minor part of the whole Ba^{2+} currents, currents were elicited by 150 ms voltage step to 0 mV from a V_h of −90 mV. (Cell 612B) (B) HWTX-I potently blocked HVA Ba^{2+} currents in the same cell, currents were elicited by 150 ms voltage step to 0 mV from a V_h of −40 mV. (C) Comparison of HWTX-I's effects on the whole Ba^{2+} currents and the HVA Ba^{2+} currents. Initial current density and that after toxin application were 14.33 ± 4.93 pA/pF and 10.08 ± 4.05 pA/pF ($n = 6$) for the whole Ba^{2+} current, 8.40 ± 4.89 pA/pF and 3.98 ± 2.07 pA/pF ($n = 6$) for the HVA Ba^{2+} current.

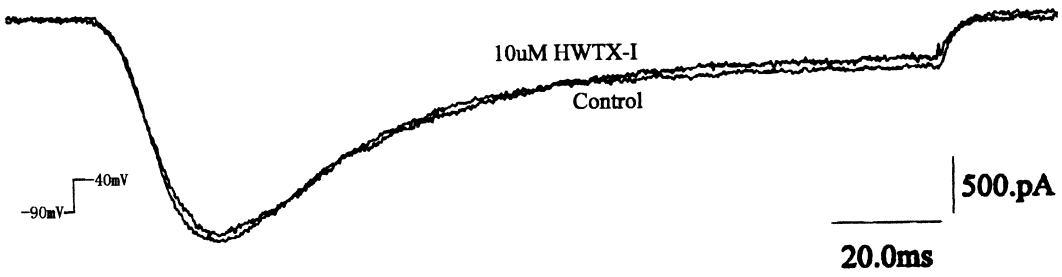

Fig. 4. HWTX-I did not influence LVA Ba^{2+} current ($n = 5$). T-type Ba^{2+} current was elicited by 100 ms voltage step to −40 mV from a V_h of −90 mV, currents were almost unaffected by 10 μM HWTX-I ($n = 5$). (Cell 41101).

MΩ and voltage errors from series resistance remaining after partial compensation were less than 5 mV. $CdCl_2$ (50 μM) was usually added at the end of the recording, which always abolished any residual inward currents that remained in toxin for cells clamped at 40 mV. However, some residual currents remained for those clamped at −90 mV (e.g., Fig. 3A, B).

2.5. Rundown problem

When studying Ca^{2+} channels, rundown of currents was a major problem. ATP and Mg^{2+} are essential to maintain stable Ca^{2+} currents, however, high concentration of Mg^{2+} may inhibit Ca^{2+} currents (Byerly and Yazejian., 1986). In our experiments, addition of 1 mM MgATP into the internal solution could well prevent rundown of Ca^{2+} currents (Fig. 1) within a period of about 1 h. Effects of toxins always took place quickly and most experiments were done within half an hour. Thus, excluding rundown problem from analysis of our experiment data would not cause much trouble.

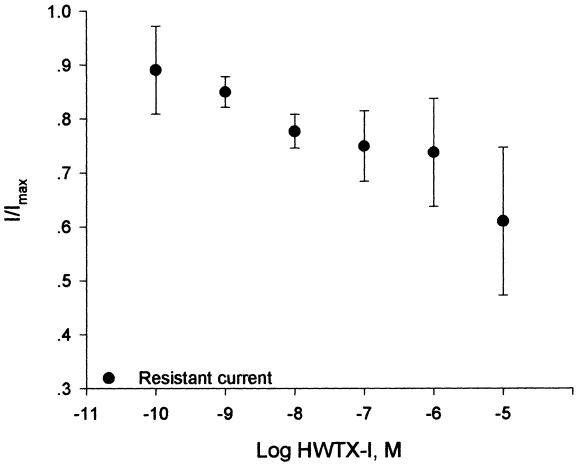

Fig. 5. Does-dependent block by HWTX-I of HVA Ba^{2+} currents. Current were elicited by 150 ms voltage step to 0 mV from a holding potential of −40 mV. Data points (mean ± SEM, 3–10 cells per point) show current relative to control. Block was determined after toxin had been applied for >2 min.

3. Results and discussion

3.1. Effect of HWTX-I on Na^+ and K^+ channels

NG108-15 cells were seldom used for studying Na^+ channel. Na^+ channel currents recorded from cells cultured with traditionally 10% serum-medium were fairly small (1–2 nA). We found that cells cultured with 5% serum-medium exhibit much larger macroscopic Na^+ channel currents (sometimes might reach 15–20 nA), and this type of Na^+ channel currents could be abolished by TTX. Low serum concentration might have induced neuronal growth, and thus raised the density of voltage-gated Na^+ channel in this cell line. HWTX-I seemed to have no significant effect on this TTX-sensitive Na^+ current or the delayed rectifier K^+ current (K_r) in this cell line (Fig. 2).

3.2. Selectivity of HWTX-I on low-voltage-activated Ca^{2+} channel and high-voltage-activated Ca^{2+} channel

There is only negligible LVA Ca^{2+} current (T-type) in undifferentiated NG108-15 cells, large Ca^{2+} currents with an amplitude of 1–5 nA could be recorded from prostaglandin E_1 differentiated Ng108–15 cells. However, Ca^{2+} channel types induced by this method are far from homogeneous. Lukyanetz (1999) suggested that for cells without neurites, T-, L-, and N-type Ca^{2+} channel components presented approximately equal contributions to the total Ca^{2+} currents, but for differentiated cells with neurites, the contributions of each components are 50, 17, 20 and 12% for N-, L-, T- type and residual Ca^{2+} currents, respectively, and the residual Ca^{2+} current is mostly Q-type (Lukyanetz, 1998). In our experiments, we always chose spherical and elliptic cells without prominent neurites, and the properties of Ca^{2+} currents obtained were more like

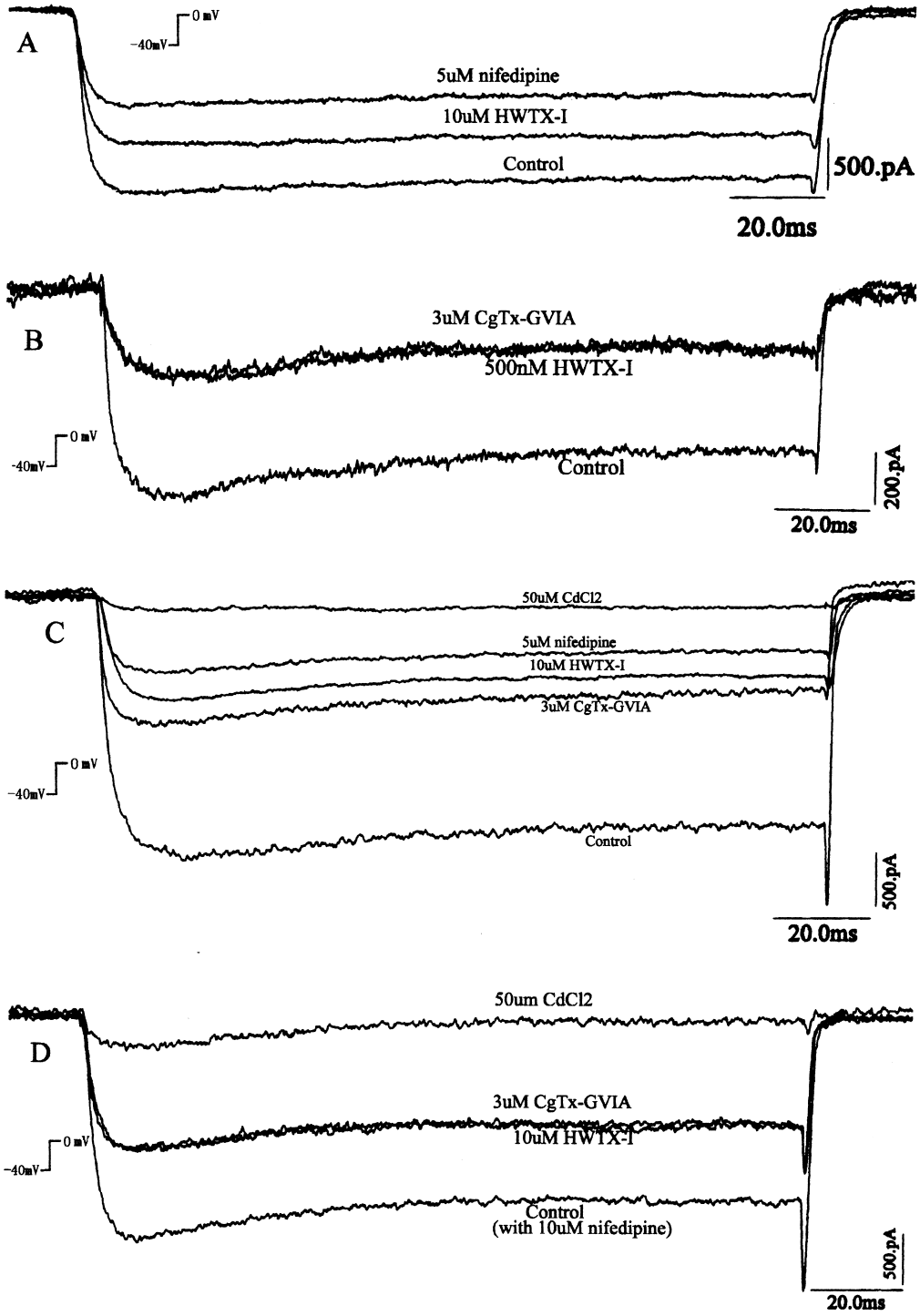

Fig. 6. HWTX-I's effects on N-type and L-type HVA Ba^{2+} current. (A) Nifedipine (5 μM) blocked another part of HVA Ba^{2+} currents after application of HWTX-I (Cell 41104, $n = 4$). (B) ω-conotoxin GVIA (3 μM) had no additional blockade on HVA Ba^{2+} current after application of HWTX-I (Cell 615, $n = 4$). (C) HWTX-I blocked none or a minor part of HVA Ba^{2+} currents in the presence of ω-conotoxin GVIA (3 μM). Nifedipine (5 μM) always blocked an additional part in that case (Cell 61101, $n = 4$). (D) HWTX-I still potently block HVA Ba^{2+} currents in the presence of nifedipine (10 μM). ω -conotoxin GVIA (3 μM) had no additional blockade after application of HWTX-I (Cell 61202, $n = 3$).

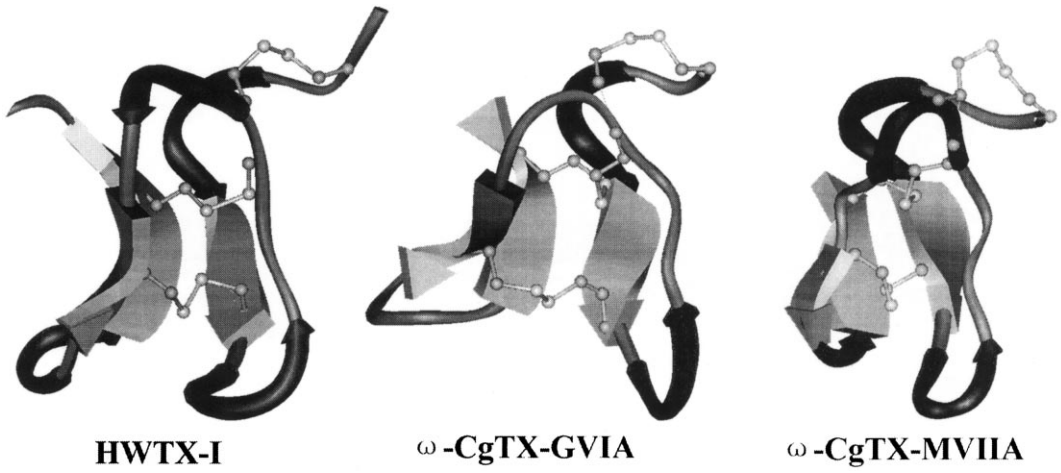

Fig. 7. The three-dimensional structures of ω-CgTX GVIA, ω-CgTX MVIIA and HWTX-I.

the former, so we would not consider Q-type Ca^{2+} current in the latter discussion.

When a cell was depolarized to 0 mV from a holding potential (V_h) of −90 mV, all components would be activated, while almost only HVA Ca^{2+} channel current was activated if the cell was depolarized from a V_h of −40 mV. When applied at 10 μM concentration, in both cases (V_h −90 and −40 mV), HWTX-I inhibited a part of Ca^{2+} currents obtained, but the block was much more potent in the later case (Fig. 3A, B). Mean resistant currents were 67.87% (45–91%, $n = 8$) and 54.08% (26–79%, $n = 9$). T-type Ca^{2+} currents obtained by depolarizing cells from −90 to −40 mV were almost unaffected by 10 μM HWTX-I, (Fig. 4). These results implied that HVA Ca^{2+} channel might be the target of HWTX-I, and it had almost no effect on LVA Ca^{2+} channel (T-type).

The inhibition of high-threshold Ca^{2+} channel by this toxin was dose dependent. In differentiated Ng108-15 cells, a fraction of about 45% was sensitive to the toxin ($EC_{50} \approx 100$ nM, Fig. 5). Toxin block was rapid at high concentration and could be slowly reversed (>10 min). It is odd that at such a relative high concentration of 10 μM HWTX-I's effects on HVA Ca^{2+} currents differed so much from cell to cell, when statistical data of ω-conotoxin GVIA's effects on HVA Ca^{2+} currents in this cell line show much similarities (0–90% block, $n = 60$) (Kasai et al., 1992). One probable reason might be the irregularity of the composition of expressed Ca^{2+} channels induced by differentiation.

3.3. Effects of HWTX-I on N-type and L-type Ca^{2+} channels

According to Kasai et al. (1992), in prostaglandin E_1 differentiated NG108-15 cells, HVA Ca^{2+} currents are mainly N-type Ca^{2+} current (sensitive to ω-conotoxin GVIA) and L-type Ca^{2+} current (sensitive to nifedipine). After HWTX-I's blockade, nifedipine inhibited another fraction of HVA Ca^{2+} currents (Fig. 6A) while ω-conotoxin GVIA had no additional effect (Fig. 6B), suggesting that N-type Ca^{2+} channel was almost completely blocked by HWTX-1. In the presence of nifedipine, HWTX-I still potently inhibited HVA Ca^{2+} current (Fig. 6D), while after ω -conotoxin GVIA's blockade, Ca^{2+} current became insensitive to HWTX-I (Fig. 6C), indicating that HWTX-I had little effect on L-type Ca^{2+} Channel. These results indicated that HWTX-I acted mainly as an N-type Ca^{2+} channel inhibitor.

The above results of whole-cell patch clamp experiments on prostaglandin E_1 differentiated NG108-15 cells can well explain our previous results that HWTX-I has presynaptic activity which inhibits the release of neurotransmitter from the nerve terminal of both, the cholinergic and the adrenergic synapse (Liang et al., 2000).

Peptides that block voltage-gated Ca^{2+} channels have also been isolated from several other animals such as marine snails (Mccleskey et al., 1987) and funnel web spider (Lin et al., 1990). Among them, ω-conotoxin MVIIA, like HWTX-I, can selectively block the N-type Ca^{2+} channel. Comparison of the three-dimensional structure shows that the two neurotoxic peptides have the same structural motif which is characterized by a triple-stranded anti-parallel β-sheet stabilized by a cystine knot (Basus et al., 1995; Qu et al., 1997). Fig. 7 illustrates the comparison of the main chains of the structure of HWTX-I, ω-CgTXGVIA and ω-CgTXMVIIA. The three molecules are very similar to each other, especially the main loop regions near the N-terminals. This result indicates that the functional similarity of the three neurotoxic peptides has its struc-

ture basis. ω-CgTXGVIA, ω-CgTXMVIIA and HWTX-I can be used as agents for further study of voltage-gated Ca^{2+} channel. It would be significant to make a further investigation in order to compare the structure and function of these three N-type Ca^{2+} channel inhibitors that are from different animals and have different amino acid sequences.

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References

Basus, V.J., Nadasdi, L., Ramaohandran, J., Miljanich, G.P., 1995. Solution structure of ω -conotoxin MVIIA using 2D-NMR spectrescopy. FEBS Lett 370, 163–169.

Byerly, L., Yazejian, B., 1986. Intracellular factors for the maintenance of calcium currents in perfused neurons from the snail, *Lymnaea Stagnalis*. J. Physiol. (London) 370, 631–650.

Harald, Reuter., 1983. Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature 301, 569–574.

Hirning, L.D., Fox, A.P., McCleskev, E.W., Oliver, B.M., Thayer, S.A., Miller, R.J., Tisen, R.W., 1988. Dominant role of N-type Ca^{2+} channels in evoked release of norepo-nephrine from sympathetic neurons. Science 239, 57–61.

Kasai, H., Neher, E., 1992. Dihydropyridine-sensitive and ω-conotoxin-sensitive calcium channels in a mammalian neuroblastoma-glioma cell line. J. physiol. (London) 488, 161–188.

Liang, S.P., Chen, X.D., Shu, Q., Zhang, Y.Q., Peng, K., 2000. The presynaptic activity of Huwentoxin-I, a neurotoxin from the venom of the spider *Selenocosmia huwena*. Toxicon 38 (9), 1237–1246.

Liang, S.P., Zhang, D.Y., Pan, X., Zhou, P.A., 1993. Properties and amino sequence of Huwentoxin-I, a neurotoxin purified from the venom of the Chinese bird spider Selenocosmia Huwena. Toxicon 31, 969–978.

Liang, S.P., Xia, Z.X., Xie, J.Y., 1997. Solid-phase synthesis of huwentoxin-I and its structure and bioactivity analysis. Science in China(C) 40, 449–457.

Lin, J.W., Rudy, B., Llinas, R., 1990. Funnel-web spider venom and a toxin fraction block calcium current expressed from rat brain mRNA in *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA 87, 4538–4542.

Mccleskey, E.W., Fox, A.P., Feldman, D.H., Crux, L.J., Olivera, B.M., Tsien, R.W., Yoshikami, D., 1987. ω-Conotoxin: Direct and persistent blockade of specific types of calcium channels in neurons but not muscle. Proc. Natl. Acad. Sci. USA 84, 4327–4331.

Miller, R.J., 1987. Multiple calcium channels and neuronal function. Science 235, 46–52.

Miller, R.J., 1992. Voltage-sensitive Ca^{2+} channels. J. Biol. Chem. 267, 1403–1406.

Mintz, I.M., Venema, V.J., Adams, M.E., Beam, B.P., 1991. Inhibition of N- and L-type Ca^{2+} channels by the spider venom toxin ω -Aga-IIIA. Proc. Natl. Acad. Sci. USA 88, 6628–6631.

Nowycky, M.C., Fox, A.P., Tsien, R.W., 1985. Three types of neuronal calcium channel with different calcium agonist sensitivity. Nature 316, 440–443.

Qu, Y.X., Liang, S.P., Ding, J.Z., Liu, X.C., Zhang, R.J., Gu, X.C., 1997. Proton nuclear magnetic resonance studies on Huwentoxin-I from the venom of the spider *Selenocosmia huwena*: 2. Three-dimentional structure in solution. J. Prot. Chem. 16, 565–574.

Watermam, S.A., 1997. Role of N-, P- and Q-type voltage-gated calcium channels in transmitter release from sympathetic neurons in the mouse isolated vas deferens. Br. J. Phamacol. 12, 393–398.

Lukyanetz, E.A., 1998. Diversity and properties of calcium channel types in NG 108-15 hybrid cells. Neuroscience 87, 265–274.

Zhou, P.A., Xie, X.J., Li, M., Yang, D.M., Xie, Z.P., Zong, X., Liang, S.P., 1997. Blockade of neuromuscular transmission by huwentoxin-I, purified from the venom of the Chinese bird spider *Selenocosmia huwena*. Toxicon 35, 39–45.