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EFFECTS OF WAGLERIN-I ON NEUROMUSCULAR TRANSMISSION OF MOUSE NERVE–MUSCLE PREPARATIONS

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M.-C. Tsai, W. H. Hsieh, L. A. Smith and C. Y. Lee. Effects of waglerin-I on neuromuscular transmission of mouse nerve–muscle preparations. *Toxicon* **33**, 363–371, 1995.—The effects of waglerin-I, a toxin from *Trimeresurus wagleri*, on neuromuscular (NM) transmission were studied on the phrenic nerve–diaphragm preparation and triangularis sterni nerve–muscle preparation of mice. The toxin (1.2–4.0 μM) reversibly inhibited the indirectly elicited twitch tension of the diaphragm and decreased the ACh-elicited muscle contracture of chronically denervated diaphragm, while the directly elicited twitch tension was not affected. The toxin reversibly decreased the amplitude of miniature endplate potential (MEPP) at 0.52 μM and endplate potential (EPP) at 1.2–4 nM. The toxin (120 nM–0.4 μM) also decreased the quantal content of EPP. The perineural waveforms were recorded with an extracellular electrode placed into the perineural sheaths of motor nerves of M. triangularis sterni. The toxin (4 μM) did not alter the amplitudes of waveforms related to sodium and potassium currents of the nerve terminal action potential, while the waveform related to calcium current was decreased. It is concluded that the toxin acts on both presynaptic and postsynaptic sites of the mouse motor endplate, and that the presynaptic effect is apparently more potent than the postsynaptic effect.

INTRODUCTION

Waglerin-I, a lethal toxin isolated from the venom of *Trimeresurus wagleri*, consists of 22 amino acid residues with a proline-rich sequence (Weinstein *et al.*, 1991). In our previous study, we found that respiratory failure, resulting from neuromuscular block, is the primary cause of death in mice, and that rats are rather resistant to waglerin-I (Lin *et al.*, 1995). However, the mechanism of action of the toxin on synaptic transmission remains unclear. The aim of this study was to elucidate the possible mode of action of the synthetic waglerin-I on neuromuscular transmission of mouse skeletal muscle.

MATERIALS AND METHODS

Experiments were carried out *in vitro* on the isolated phrenic-nerve hemidiaphragm (Bülbring, 1946) and the triangularis sterni muscle (McCardle *et al.*, 1981) of mice (NIH-ICR) (18–25 g) of either sex. The diaphragm was immersed in a physiological salt solution containing (mM): NaCl 135.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, $NaHCO_3$ 15.0, Na_2HPO_4 1.0 and glucose 11.0. The bath was maintained at 37 ± 1°C and continuously bubbled with a

gas mixture composed of 95% O_2 and 5% CO_2, which maintained the pH between 7.1 and 7.3. The triangularis sterni muscle was immersed in a physiological salt solution containing (mM): NaCl 115, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.0, $NaHCO_3$ 25, Na_2HPO_4 1.0 and glucose 11. The bath was maintained at room temperature (22–25°C) and continuously bubbled with a gas mixture as described previously (Penner and Dryer, 1986; Tsai and Chen, 1991).

The phrenic nerve of the diaphragm was stimulated with pulses of 0.05 msec at 0.2 Hz at a voltage greater than that required to elicit a maximum twitch by a pair of platinum electrodes connected to a Digitimer D4030 with a DS2 isolated stimulator (Tsai *et al.*, 1992). Direct stimulation of the muscle was achieved at a voltage greater than that required to elicit a maximum twitch by applying rectangular pulses of 1.0 msec duration at a rate of 0.2 Hz through a pair of platinum electrodes placed at the base of the diaphragm near the intercostal muscles. The twitch tension was recorded by a Grass FT03 force displacement transducer connected to a Grass model 7E polygraph or to a Gould UC2 transducer connected to a Gould 2200S recorder (Tsai *et al.*, 1992).

For the denervation experiment, mice were anaesthetized with pentobarbital sodium (30 mg/kg, i.p.) and the left phrenic nerve was removed at the cervical portion under aseptic conditions. After 9–14 days of denervation, the diaphragm was removed and immersed in the physiological salt solution as described above.

For intracellular recording, the muscle was fixed on a paraffin-lined plate in a 20 ml bath with a planoconvex lens at its centre, which was immersed in a bath at 30 ± 0.5°C. The frequency of miniature endplate potentials (MEPP) was recorded 20 sec after microelectrode penetration. For recording the endplate potential (EPP), the phrenic nerve was stimulated at 0.2 Hz. All recordings were made from surface fibres using glass microelectrodes filled with 3 M KCl (resistance 5–20 MΩ) (Tsai and Chen, 1991). The quantal content of the EPP was measured either by the failure method or by the measurement of the ratio of EPP/MEPP. The preparation was incubated in a physiological salt solution containing 12–15 mM $MgCl_2$ or tubocurarine (3 μM) as described in the text.

For checking the concentration of waglerin-1 that inhibited 50% of the twitch tension, amplitudes of EPP, MEPP and quantal content of EPP, the linear regression line with 95% confidence interval was drawn using Sigma Plot.

For recording the perineural waveforms, the isolated triangularis sterni muscle preparation was visualized at 400 × magnification using a Zeiss microscope equipped with Normarski interference contrast optics. Signals following nerve stimulation through a suction electrode were recorded inside the nerve bundles (containing 2–4 nerve fibres) using glass microelectrodes filled with 0.5 M NaCl (resistance 4–10 MΩ). The reference electrode was a chloride silver wire in the recording chamber. The potential difference between the silver/silver chloride reference electrode and the recording electrode was measured by a high impedance unity gain electrometer, displayed on a dual beam storage oscilloscope (Tektronix 5441) and Gould digital storage oscilloscope 450 (Tsai and Chen, 1991).

The Student's t-test was used for statistical analysis of the effects of the toxin on the mean amplitudes of twitch tension, EPP and MEPP and quantal content, and resting potential of the muscles; a paired Student's t-test was used for analysis of the effect of the toxin on the perineural waveforms, $P < 0.05$ indicating significance.

Drugs

Tubocurarine and procaine were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). 3,4-Diaminopyridine was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Tetraethylammonium chloride was purchased from Merck-Schuchardt (Munich, F.R.G.).

Toxin

Waglerin-I was synthesized with a Model 431A instrument from Applied Biosystems (Foster City, CA, U.S.A.) as described previously (Schmidt *et al.*, 1992).

RESULTS

Effect of waglerin-I on mouse phrenic nerve–diaphragm twitch tension

Waglerin-I inhibited the indirectly elicited twitch tension of the mouse diaphragm in a concentration-dependent manner (Fig. 1). After 1 hr of incubation in 1.2 and 2.4 μM waglerin-I, the twitch tension decreased to $81.9 \pm 8.5\%$ (mean $\pm$ S.E.M., $n = 5$) and $48.2 \pm 11.8\%$ ($n = 5$) of control, respectively. The twitch tension was completely blocked in 17.5 ± 2.5 min ($n = 5$) after 4 μM waglerin-1 application. The concentration of waglerin-I that inhibited 50% of the twitch tension was 2.2 μM (Figs 2 and 3). In contrast to the indirectly elicited contraction, the response to direct stimulation was unaffected. The effect of waglerin-I on the indirectly elicited twitch tension was reversible. After 20 min of washing, the twitch tension blocked by waglerin-I recovered to control levels.

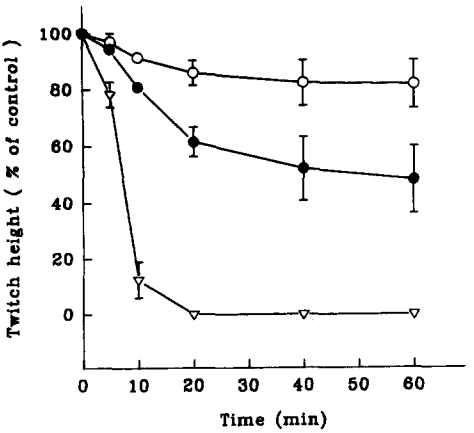

Fig. 1. Indirectly elicited isometric twitch tension of the mouse diaphragm muscle, expressed as percentage of control tension (100%), is plotted against time in min after toxin application. Each point is the mean ± S.E.M. of 3–6 preparations. The concentrations of waglerin-I are as follows: ○, 1.2 μM; ●, 2.4 μM; △, 4 μM.

Effect on the resting membrane potential of mouse diaphragm

The resting membrane potential of normal mouse diaphragm in 15 preparations tested was -72.3 ± 1.5 mV (mean ± S.E.M.). After 90 min of incubation in 4 μM waglerin-I, it was -72.1 ± 1.7 mV ($n = 14$). Thus, waglerin-1 had no effect on the resting membrane potential of the mouse diaphragm.

Effects of waglerin-I on the action potential of muscle elicited by indirect stimulation

Figure 2 shows that 4 μM waglerin-I abolished the indirectly elicited action potential of muscle. After 4 min of incubation of waglerin-I, no action potential could be recorded; only occasionally were EPPs recorded (Fig. 2b). After 10 min of incubation, both EPP and muscle action potential elicited by EPP were abolished.

Effect of waglerin-I on the EPP of mouse diaphragm

The effect of waglerin-I on the amplitude of EPP is shown in Fig. 4. In the preparation pretreated with tubocurarine (3 μM), the mean EPP amplitude was 3.4 ± 0.3 mV ($n = 6$).

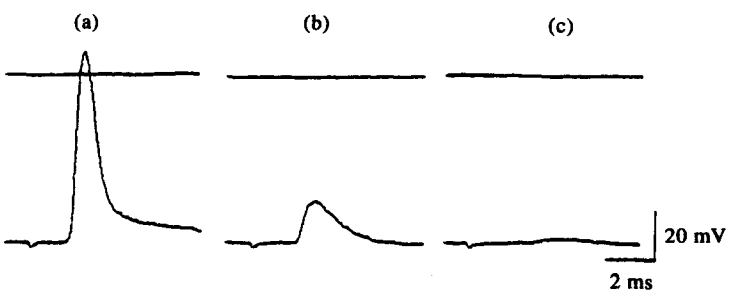

Fig. 2. Effect of waglerin-I on the muscle action potential elicited by indirect stimulation. a: Control; b: 4 min, and c: 10 min after waglerin-I (4 μM) application. Note that waglerin-I completely abolished the muscle action potential elicited by indirect stimulation.

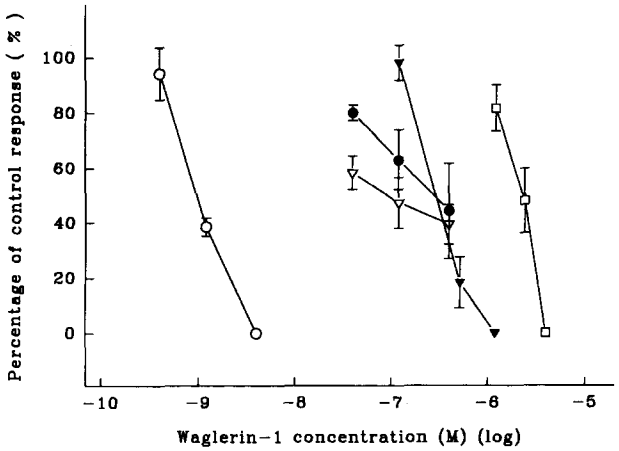

Fig. 3. The inhibitory effects of different concentrations of waglerin-I on the EPP (○), the quantal content of EPP (●) by the ratio of EPP/MEPP, and △ by failure method), the amplitude of the MEPP (▲) and the indirectly elicited twitch tension (□). Each point is the mean ± S.E.M. of 3–6 preparations. Where no bar is shown, the S.E.M. was smaller than the symbol.

At a concentration as low as 1.2 nM, waglerin-I decreased the amplitude of EPP to 1.3 ± 0.1 mV ($n = 6$) 10 min after toxin application. The EPP was abolished at a higher concentration (4 nM) in 10 min (Fig. 4d). The concentration of waglerin-I that inhibited 50% of EPP was 1.9 nM (Fig. 3). The effect of waglerin-I on the amplitude of EPP was reversible. After 30 min of washing, some amplitudes of EPPs recovered to control levels. The mean EPP recovered to 2.4 ± 0.5 mV ($n = 4$) 30 min after washing (Fig. 4e).

Effects on frequency and amplitude of MEPP in mouse diaphragm

The effects of various concentrations of waglerin-I on the amplitude of MEPP in the mouse diaphragm are shown in Fig. 3. Waglerin-I, at 0.12 μM, had no effect on the amplitude of MEPP. When the concentration of toxin was increased to 0.52 μM, the amplitude of MEPP was decreased from 0.6 ± 0.05 mV (mean ± S.E.M., $n = 28$) to 0.1 ± 0.05 mV after 10 min of incubation. The amplitude of MEPP recovered to

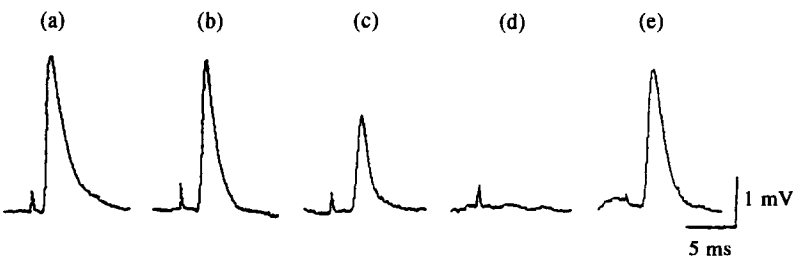

Fig. 4. Effects of waglerin-I on the endplate potential (EPP) of mouse diaphragm in the presence of D-tubocurarine (3 μM).
a: Control; b, c, and d: 10 min after waglerin-I 0.4, 1.2, and 4.0 nM application, respectively; e: endplate potential from the same area after 30 min of washing. Each EPP presented was the average of 16 signals.

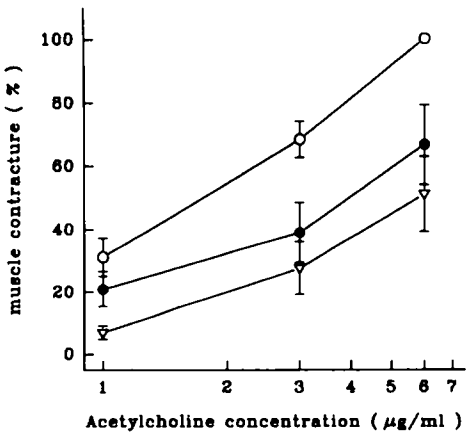

Fig. 5. Concentration–response relationship of the muscle contracture induced by ACh in the chronically denervated mouse diaphragm.
The preparations were incubated in normal physiological solution (○) and 5 min after treatment with waglerin-I 1.2 (●) and 4 μM (△), respectively. Each point is the mean ± S.E.M. of 3–6 preparations.

0.5 ± 0.06 mV after 30 min of washing. The concentration of waglerin-I that inhibited 50% of the amplitude of MEPP was 0.52 μM (Fig. 3).

Waglerin-I also decreased the frequency of MEPP. Ten minutes after 0.12 μM waglerin-I treatment, the frequency of MEPP was decreased from 1.0 ± 0.3 Hz ($n = 5$) to 0.7 ± 0.2 Hz ($n = 4$).

Effect of waglerin-I on quantal content of EPP

The effect of waglerin-I on the quantal content of the EPP, as measured by either the ratio of EPP/MEPP or the failure method, is shown in Table 1. The quantal content of EPP was decreased significantly 10 min after toxin administration at 0.12–0.4 μM. The concentration of waglerin-I that inhibited 50% of the quantal content of EPP, measured by either the failure method or the ratio of EPP/MEPP, was about 0.29–0.31 μM.

Table 1. Effects of various concentrations of waglerin-I on the quantal content (m) of EPP of mouse diaphragm

Method tested	Quantal content (m)	
	EPP/MEPP	No. of failure
Control	2.44 ± 0.16	2.99 ± 0.33
Waglerin-I (nM)		
40	1.96 ± 0.07	1.74 ± 0.19*
120	1.54 ± 0.27*	1.42 ± 0.28*
400	1.08 ± 0.43*	1.18 ± 0.21*

The preparations were pretreated with 13 mM $MgCl_2$. Each value is the mean ± S.E.M. from 3–6 preparations.
*$P < 0.05$ compared with control.

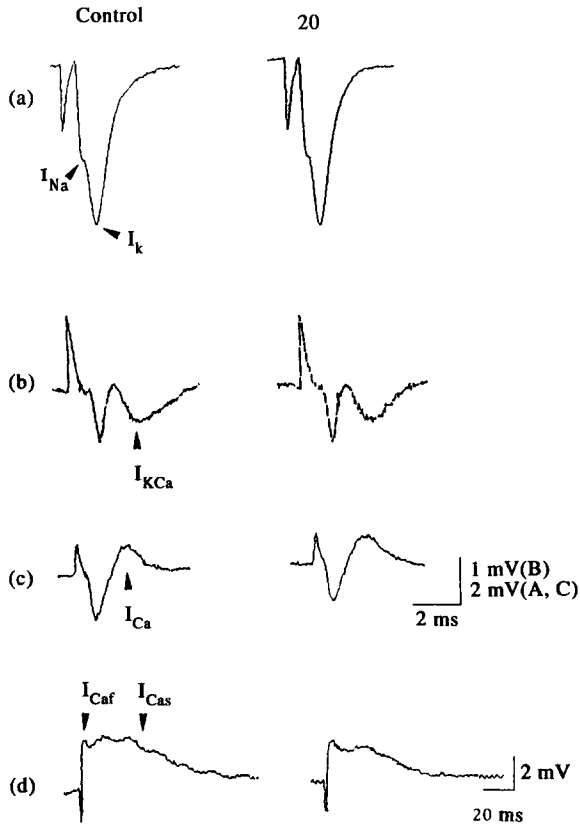

Fig. 6. The effects of waglerin-I (4 μM) on perineural waveforms of a motor nerve terminal of mouse.

Each waveform presented was the average of eight signals. At control: waveforms before waglerin-I treatment; at 20: waveforms after 20 min of incubation in waglerin-I. (a) The preparation was incubated in normal physiological solution containing 25 μM D-tubocurarine. Note that the perineural waveform showed a biphasic negativity which corresponded to sodium current (I_{Na}) and potassium current (I_K) of the nerve terminal. (b) The effect of waglerin-I on calcium-activated potassium current (I_{KCa}) of a motor nerve terminal of mouse. The perineural waveforms were recorded in the same area of the nerve terminal. B-control: perineural waveform recorded after 20 min of addition of 40 μM tubocurarine and 500 μM 3,4-DAP. B-20: perineural waveform 20 min after waglerin-I (4 μM) application. Note that I_{KCa} could still be recorded after waglerin-I application. (c) The effects of waglerin-I on the slow potassium current of a motor nerve terminal of mouse. C-control: perineural waveform after suppression of the fast potassium current and calcium activated potassium current by tetraethylammonium (TEA). C-20: signal recorded in the same area of the nerve terminal 20 min after waglerin-I (4 μM) application. D-Tubocurarine (40 μM) and TEA (30 mM) were added throughout the experiment. (d) The effect of waglerin-I on presynaptic calcium currents in the motor nerve terminal of mouse. D-Control: example of I_{Ca} of a motor nerve terminal after the suppression of potassium currents by TEA (1 mM) and 3,4-DAP (50 μM) and increasing the extracellular calcium concentration to 6 mM. D-20: calcium currents recorded in the same area 20 min after waglerin-I application.

Effects on the chronically denervated mouse diaphragm

The effects of various ACh concentrations on the chronically denervated mouse diaphragm are shown in Fig. 5. After waglerin-I (1.2–4 μM) application for 5 min, the ACh-induced muscle contracture was significantly decreased.

Effects of waglerin-I on perineural waveforms

Figure 6a shows a perineural waveform following supramaximal nerve stimulation. It consists of a predominantly biphasic negativity which is often preceded by a small positivity. This waveform closely resembles focally recorded signals obtained at the transition between myelinated axon and non-myelinated terminal, where evidence has been presented that the first negativity is due to Na^+ influx (I_{Na}) and the second negative phase corresponds to the current generated by potassium efflux (I_K) in the nerve terminals (Bright and Mallart, 1982; Mallart, 1985*a*). Waglerin-I (4 μM) did not have a significant effect on the perineural waveform components of sodium and potassium in the nerve terminal.

Effects of waglerin-I on calcium activated potassium current

Pharmacological evidence has shown that the ion channels traversed by this current in mouse motor endings (Mallart, 1985*a*) share many properties with the large conductance of calcium channels presented in T-tubules and myotubes (Latorre, 1986).

For investigations of the calcium activated potassium current (I_{KCa}), 500 μM 3,4-DAP was added to the bath solution (Mallart, 1985*b*). This concentration ensured maximal responses and consistently revealed the typical triphasic signals as shown in Fig. 6b. The signal component of the I_{KCa} was not affected after application of 4 μM waglerin-I. The result indicates that the toxin had no effect on the I_{KCa} of nerve terminal current.

Effects on slow TEA-resistant potassium current

TEA (30 mM) failed to elicit full calcium plateau responses even if external calcium was increased to 6 mM. Prolonged calcium responses could only be obtained by subsequent addition of 3,4-DAP (200 μM) (Tsai and Chen, 1991). This was taken as evidence for the presence of a TEA-resistant K^+ current in mammalian motor nerve terminals (Penner and Dreyer, 1986). We studied the sensitivity of this current to waglerin-I by looking for the ability of this peptide to promote full calcium plateau in 30 mM TEA-treated preparations. As shown in Fig. 6c, a calcium plateau could not be elicited after adding 4 μM waglerin-I. However, the calcium plateau was amplified and prolonged by subsequent addition of 3,4-DAP (data not shown). The prolonged plateau calcium current response after addition of 3,4-DAP was not altered after repetitive washing of the toxin. These results indicate that waglerin-I (4 μM) had no effect on the slow TEA-resistant potassium current.

Effects of waglerin-I on presynaptic calcium currents

TEA and 3,4-DAP are potassium channel blockers. A combination of both TEA and 3,4-DAP gives rise to a large positive deflection of the presynaptic current, which is blocked by Cd^{2+}, indicating that its underlying cause is a calcium current (Penner and Dreyer, 1986).

There are two different presynaptic calcium currents in mouse motor nerve terminal. The fast positive signal component (fast calcium current, I_{Caf}) is attributed to the voltage-dependent calcium channel, responsible for the initiation of transmitter release. The slow positive signal component (slow calcium current, I_{Cas}) also depends on extracellular calcium concentration, although its physiological role remains unknown (Penner and Dreyer, 1986).

The effects of 4 μM waglerin-I on the waveforms related to calcium currents are shown in Fig. 6d. Waglerin-I decreased the waveforms 5 min after toxin administration. After

20 min of incubation, both waveforms relating to fast and slow calcium currents in the nerve terminal were decreased to 78.9 ± 3.0% ($n = 4$) and 61.3 ± 3.3% ($n = 4$) of control, respectively. It seems that waglerin-I significantly decreased the calcium components of the nerve terminal.

DISCUSSION

The toxin isolated from *Trimeresurus wagleri* venom has been suggested to be either a non-neurotoxin (Tan and Tan, 1989), or a vasoactive peptide or neurotoxin (Schmidt *et al.*, 1992; Weinstein *et al.*, 1991). In the present study, the effects of waglerin-I, a synthetic toxin, on synaptic transmission were studied in isolated mouse diaphragm and triangularis sterni muscles, using pharmacological and electrophysiological methods. We found that waglerin-I reversibly inhibited the indirectly elicited twitch tension of mouse diaphragm. This effect was not due to the depolarization of the muscle membrane because waglerin-I did not alter the resting membrane potential of the muscle. Waglerin-I also blocked the muscle action potential elicited by indirect stimulation, whereas it had no effect on the directly elicited twitch tension of the diaphragm. These results suggest that waglerin-I acts on the synaptic transmission of the motor endplate.

Waglerin-I reversibly decreased the amplitude of the endplate potential at a concentration as low as 1.2 nM in a tubocurarine-paralysed preparation. The toxin also decreased the ACh-induced muscle contracture in the chronically denervated muscle at a much higher concentration (1.2 μM). These findings suggest that waglerin-I blocks the ACh receptor ion channel complex of the motor endplate. At 0.12 μM, waglerin-I decreased significantly the frequency of miniature endplate potentials. At this concentration, however, waglerin-I did not alter the amplitude of miniature endplate potentials. These results suggest that waglerin-I has a presynaptic effect on the transmitter-releasing process. The presence of a presynaptic effect of waglerin-I was further supported by the finding that waglerin-I decreased the quantal content of the EPP. It is interesting to note that waglerin-I decreased 50% of the amplitude of EPP (IC_{50}) at 1.9 nM in the tubocurarine-paralysed preparation, whereas it decreased 50% of the amplitude of MEPP (IC_{50}) at 0.52 μM. It appears that the amplitude of EPP was much more susceptible to the toxin. The results might be due mainly to the synergistic effects of the toxin on both the presynaptic transmitter-releasing process and the postsynaptic ACh receptor blocking action. It seems that the presynaptic effect of the toxin is more potent than its postsynaptic effect.

The mode of action of waglerin-I on the presynaptic transmitter-releasing process was further studied on extracellularly recorded perineural waveforms. We found that waglerin-I had no effect on the components of waveforms associated with the sodium and potassium currents of the motor nerve terminal. It also had no effect on the waveforms associated with the calcium-activated potassium current or the slow potassium current. However, it significantly decreased the waveform associated with the calcium current of the nerve terminal. It is suggested that waglerin-I may act on the calcium channel of the nerve terminal. This effect is apparently not potent enough to explain the presynaptic action of this toxin. Waglerin-1 may have other presynaptic effects besides the effect on the calcium current of the nerve terminal action potential. The effect of waglerin-1 on the signal transduction process resulting in the transmitter-releasing process is a subject for future studies.

Based on the results that waglerin-I acts on both presynaptic and postsynaptic sites of the motor endplate, the blocking effect of waglerin-I on the synaptic transmission is

apparently due to the synergistic effect of both presynaptic and postsynaptic inhibitory actions of the toxin. In conclusion, waglerin-I is a new type of snake neurotoxin which acts on both presynaptic and postsynaptic transmission.

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REFERENCES

Brigant, J. L. and Mallart, A. (1982) Presynaptic currents in mouse motor endings. *J. Physiol. (Lond.)* **333,** 619–636.

Bülbring, E. (1946) Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Br. J. Pharmac.* **1,** 38–61.

Latorre, R. (1986) The large calcium-activated potassium channel. In: *Ion Channel Reconstruction*, p. 431 (Miller, C., Ed.). New York: Plenum Press.

Lin, W. W., Smith, L. A. and Lee, C. Y. (1995) A study on the cause of death due to waglerin-I, a toxin from *Trimeresurus wagleri. Toxicon* **33,** 111–114.

Mallart, A. (1985*a*) Electric current flow inside perineurial sheaths of mouse motor nerves. *J. Physiol. (Lond.)* **368,** 565–575.

Mallart, A. (1985*b*) A calcium-activated potassium current in motor nerve terminals of the mouse. *J. Physiol. (Lond.)* **368,** 577–591.

McArdle, J. J., Angaut-Petit, D., Mallart, A., Bournaud, R., Faille, L. and Brigant, J. L. (1981) Advantages of the triangularis sterni muscle of the mouse for investigations of synaptic phenomena. *Neurosci. Meth.* **4,** 109–115.

Penner, R. and Deyer, F. (1986) Two different presynaptic calcium currents in mouse motor nerve terminals. *Pflugers Arch.* **406,** 190–197.

Schmidt, J. J., Weinstein, S. A. and Smith, L. A. (1992) Molecular properties and structure function relationships of lethal peptides from venom of Wagler's pit viper, *Trimeresurus wagleri. Toxicon* **30,** 1027–1036.

Tan, N. H. and Tan, C. S. (1989) The enzymatic activities and lethal toxins of *Trimeresurus wagleri* (speckled pit viper) venom. *Toxicon* **27,** 349–357.

Tsai, M. C. and Chen, M. L. (1991) Effects of brevetoxin-B on motor nerve terminals of mouse skeletal muscle. *Br. J. Pharmac.* **103,** 1126–1128.

Tsai, M. C., Su, J. L., Fan, S. Z. and Cheng, C. Y. (1992) Effects of 3,3-dipyridymethyl-1-phenyl-2-indolinone on the neuromuscular transmission in the rodent skeletal muscles. *Neuropharmacology* **31,** 89–94.

Weinstein, S. A., Schmidt, J. J., Bernheimer, A. W. and Smith, L. A. (1991) Characterization and amino acid sequences of two lethal peptides and structure–function relationships of lethal peptides from venom of Wagler's pit viper. *Trigeresurus wagleri. Toxicon* **29,** 227–236.