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WWamide-1, -2 and -3: novel neuromodulatory peptides isolated from ganglia of the African giant snail, *Achatina fulica*

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Three novel neuropeptides, isolated from ganglia of the African giant snail, *Achatina fulica*, were named WWamide-1, -2 and -3 These substances were biologically active heptapeptide amides with a Trp residue at both the N- and C-termini. WWamide-1, which displayed an inhibitory activity on a central neuron of the snail, exhibited peripherally modulatory effects on muscular contractions of not only the gut and other tissues of the snail but also certain tissues of other molluscs.

Molluscan neuropeptide; Neuromodulatory effect, *Achatina fulica*

1. INTRODUCTION

It seems general that invertebrate muscles are regulated by multiple types of neurons which release multiple species of neuromediators. For instance, the anterior byssus retractor muscle (ABRM) of the bivalve mollusc, *Mytilus edulis*, has been suggested to be controlled by at least seventeen peptide substances in addition to five biogenic amines [1]. Therefore, it has been indispensable to isolate and characterize neuroactive peptides for understanding neuronal control of invertebrate muscles. Since the isolation of FMRFamide in 1977 by Price and Greenberg [2], a large number of neuropeptides have been isolated from molluscs. According to the structure and action of the peptides, most of them could be classified into several groups such as *Mytilus* inhibitory peptide (MIP), myomodulin-CARP, buccalin and small cardioactive peptide families [3]. However, certain peptides were found to have unique structural features. The most notable examples were achatin-I [4] and fulicin [5] which were isolated from ganglia of the African giant snail, *Achatina fulica*. Each peptide contained a D-amino acid residue at the second position from N-terminus. We have been screening neuropeptides in the ganglia of *A. fulica* since 1990 using the electrically evoked phasic contraction of ABRM of *M. edulis* as a bioassay system. In the present experiments, three novel homologous peptides with distinct structural features were isolated. Each peptide had a Trp residue at both the N- and C-termini. We report the structures and neuromodulatory effects of the peptides on certain tissues of molluscs.

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Abbreviations CARP, catch-relaxing peptide; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; SIMS, secondary ion mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; ACh, acetylcholine.

2. MATERIALS AND METHODS

2.1 *Purification*

Cerebral and suboesophageal ganglia of *A. fulica* were excised from 1,250 specimens. The ganglia were homogenized and extracted with 100% acetone at 0°C. After centrifugation at 16,000 × *g* for 40 min, the pellet was extracted twice with 80% acetone. Extracts were pooled and condensed into 1 ml in vacuo prior to dilution with 0.1 N HCl After repeating centrifugation (16,000 × *g*) of the suspension, the supernatant was passed through five Sep-Pak C18 cartridges in series. The retained material was washed with 0 1% TFA and eluted with 0 1% TFA-methanol before the eluent was condensed in vacuo. The residue was dissolved in 0.1% TFA and fractionated by HPLC. Each fraction was assayed on the phasic contractions evoked by electrical stimulations of the ABRM of *M. edulis* Experimental procedures of the assay were similar to those described previously [6]. On elution by a reversed-phase column (Asahipak ODP-50) with a 120-min gradient of 0 to 60% acetonitrile in 0 1% TFA, four inhibitory peaks were obtained. The third peak was divided into three parts by a strong acid ion-exchange column (TSKgel SP-5PW) with a 70-min gradient of 0 to 0.7 M NaCl in 10 mM phosphate buffer (pH 6 9). Each part was further separated by a reversed-phase column (TSKgel ODS-80T_M) with a 50-min gradient of 18 to 28% acetonitrile in 0.1% TFA, and three inhibitory peaks were obtained They were further purified by the same column with isocratic elution ratios of 23, 23.5 and 22.5% acetonitrile in 0.1% TFA and were named WWamide-1, -2 and -3, respectively. WWamide-1, -2 and -3 were thus purified.

2.2 *Structure determination and synthesis*

WWamide-1, -2 and -3 were sequentially determined by an amino acid analyzer (Tosoh CCP-8000) and a protein sequencer (Shimadzu

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PSQ-1 protein sequencer). Their molecular weights were determined by liquid SIMS (Hitachi M-80B) and FAB-MS (JEOL JMS-HX110/110A). According to the anticipated nomenclature, WWamide-1 was synthesized by a conventional solid-phase method (Applied Biosystems Inc. Model 430A peptide synthesizer). The WWamide-1 preparation was subjected to structural confirmation, and its biological activities were examined.

2.3. *Biological activity*

The activities of WWamide-1 were examined on various neurons and muscles. The former cells were composed of the periodically oscillating neuron (PON), tonically autoactive neuron (TAN), dorsal right cerebral distinct neuron (d-RCDN), B1 and B4 neurons of *A. fulica* [7,8]; and the latter tissues involved the crop [9], penis retractor and radula retractor muscles [5] of *A fulica*, ABRM of *M. edulis* [6] and radula protractor muscle of *R. thomasiana* [10]. Electrical activities of the neurons were recorded by an intracellular microelectrode method [8]. Methods for stimulating each muscle and recording the tension were described in previous papers [5,6,9,10], respectively. FMRFamide and ACh were used to elicit contractions of ABRM, whereas ACh and Glu were employed for the radula protractor muscle of *R thomasiana*. The composition of artificial seawater (ASW) used for ABRM of *M edulis* was as follows: 445 mM NaCl, 55 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM KCl, 10 mM Tris-HCl (pH 7.8). However, physiological saline used for *A. fulica* was of a different composition: 61 mM NaCl, 3.3 mM KCl, 10.7 mM $CaCl_2$, 13 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES-NaOH (pH 7.5). Two types of ASW containing low (20 mM) and high (100 mM) concentrations of $MgCl_2$ were used for the muscle of *R. thomasiana*.

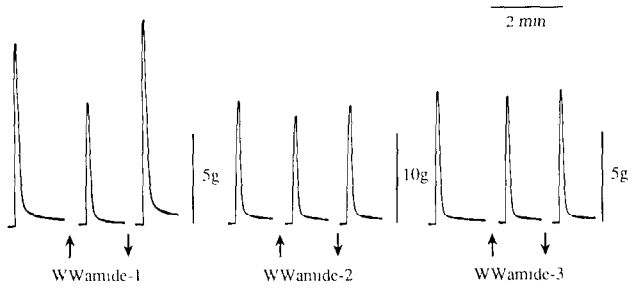

Fig. 1. Effects of native WWamides on the phasic contractions of ABRM of *M edulis*. Dose of WWamide-1 and -2 applied to the muscle corresponded to 24 ganglia/ml medium, and that of WWamide-3 was adjusted to 3 ganglia/ml medium. The peptides inhibited phasic contractions of the muscle evoked by direct repetitive electrical stimulation (15 V, 3 ms, 10 Hz, 50 pulses) at 10-min intervals.

3. RESULTS AND DISCUSSION

Purified WWamide-1, -2 and -3 showed an inhibitory effect on the phasic contractions of ABRM (Fig. 1). The results of amino acid and sequence analyses, liquid SIMS and FAB-MS are shown in Table I. Peaks of the molecular ions illustrated the presence of a C-terminal amide in the peptides. The primary structures inferred

Table I

Structural analysis of the purified peptides

	Amino acid analysis		Sequence analysis			MS $(M + H)^+$
		pmol	Cycle #		pmol	
WWamide-1						
	Ser	251.3 (0.85)	1	Trp	69.0	964 (observed)[a]
	Glx	297.7 (1.00)	2	Lys	94.1	964 (calculated)[c]
	Val	201.4 (0.68)	3	Glu	49.9	
	Met	290.8 (0.98)	4	Met	97.1	
	Lys	296.3 (1.00)	5	Ser	97.5	
			6	Val	63.9	
			7	Trp	21.3	
WWamide-2						
	Ser	24.8 (0 86)	1	Trp	13.2	992 (observed)[b]
	Glx	29.9 (1.04)	2	Arg	5.0	992 (calculated)[c]
	Val	16.7 (0.58)	3	Glu	9.9	
	Met	24.9 (0.86)	4	Met	24 6	
	Arg	28 8 (1.00)	5	Ser	26.4	
			6	Val	12.6	
			7	Trp	2 8	
WWamide-3						
	Ser	36.5 (0.88)	1	Trp	15.3	963 (observed)[b]
	Glx	45.5 (1.10)	2	Lys	25.1	963 (calculated)[c]
	Val	28.0 (0.68)	3	Gln	17.9	
	Met	40.9 (0.99)	4	Met	19.1	
	Lys	41.4 (1.00)	5	Ser	28.4	
			6	Val	17.5	
			7	Trp	3.0	

[a]Liquid SIMS; [b]FAB-MS; [c]calculated for the C-terminal amide peptides.

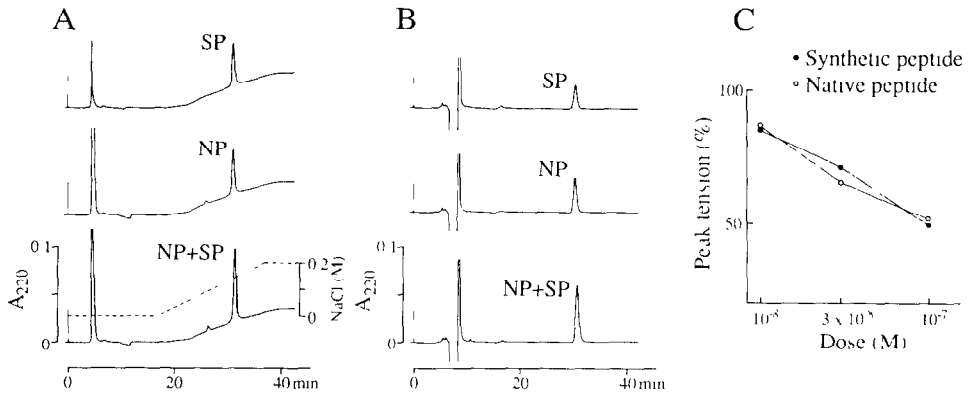

Fig. 2. HPLC profiles and dose–response relationship of the native (NP) and synthetic (SP) WWamide-1. (A) Elution with the cation exchange column was performed at a 20-min gradient of 0 to 0.2 M NaCl in 10 mM phosphate buffer (pH 6.9) (B) The reversed-phase column was eluted isocratically with 23.5% acetonitrile in 0.1% TFA (pH 2.2). (C) Dose–response relationship for native and synthetic WWamide-1 on the phasic contractions of ABRM in response to repetitive electrical stimulations (15 V, 3 ms, 10 Hz, 50 pulses, 10-min interval). The decreased phasic contraction was expressed as a percentage of the control peak tension.

by the analysis were as follows: H-Trp-Lys-Glu-Met-Ser-Val-Trp-NH_2 (WWamide-1), H-Trp-Arg-Glu-Met-Ser-Val-Trp-NH_2 (WWamide-2) and H-Trp-Lys-Gln-Met-Ser-Val-Trp-NH_2 (WWamide-3). As each peptide had a Trp residue at both the N- and C-termini, the peptides were named WWamide-1, -2 and -3. The sequences were not related to those of any known peptides. The structure and biological activities between the native and synthetic WWamide-1 were compared, and confirmed by observing their HPLC illustrations and effects on ABRM (Fig. 2).

The percentage of Trp contained in these peptides and proteins was the least among the 20 proteinous amino acids. However, the functional roles of Trp residues are extremely important in keeping conformation and thus exhibiting biological activity. Activities of the synthetic WWamide-1 on several neurons (PON, TAN, d-RCDN, B1 and B4) and three muscles (crop, penis retractor and radula retractor muscles) of *A. fulica* were examined. WWamide-1 inhibited the neuronal spike with a remarkable hyperpolarization in the d-RCDN, which is one of a pair of cerebral neuron cells, at 2×10^{-5} M (Fig. 3A). However, the other neurons did not respond to the peptide at all. Since brain peptides have been known to affect activities of the gut, effects of the peptide on snail crop were investigated. WWa-

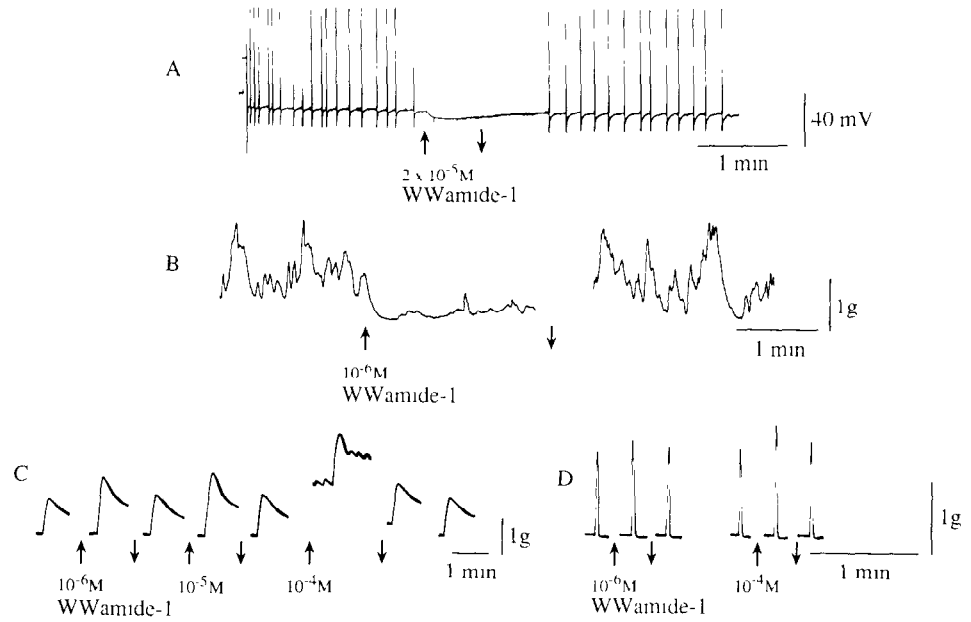

Fig 3. Effects of WWamide-1 on a neuron and muscles of *A. fulica*. (A) Effect of 2×10^{-5} M WWamide-1 on the spontaneous activity of d-RCDN. (B) Effect of 10^{-6} M WWamide-1 on the spontaneous contractions of crop. (C) Dose-dependent activities of WWamide-1 on the tetanic contractions of penis retractor muscle evoked by repetitive electrical stimulations (17 V, 0.8 ms, 40 Hz for 1 s). (D) Dose-dependent potentiations of WWamide-1 on the tetanic contractions of radula retractor muscle evoked by repetitive electrical stimulations (12 V, 0.6 ms, 40 Hz for 1 s).

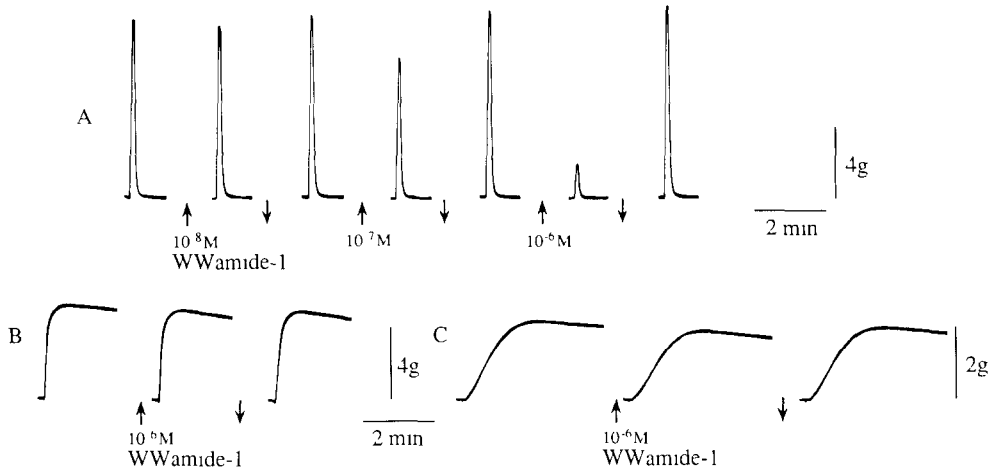

Fig. 4. Effects of WWamide-1 on ABRM of *M. edulis*. (A) Dose-dependent inhibitions of WWamide-1 on the phasic contractions in response to repetitive electrical stimulations (15 V, 3 ms, 10 Hz, 50 pulses, 10-min interval). (B) Effect of 10^{-6} M WWamide-1 on muscular contractions induced by 10^{-5} M ACh. (C) Effect of 10^{-6} M WWamide-1 on contractions induced by 10^{-6} M FMRFamide.

mide-1 inhibited spontaneous contractions of the crop with a threshold of 10^{-5} M (Fig. 3B). At 10^{-6} M or higher, WWamide-1 potentiated the electrically evoked tetanic contractions of the penis retractor and radula retractor muscles (Fig. 3C,D). These results suggest that WWamide-1 may play a role in the central and peripheral nervous systems of snail. WWamide-1 may be a useful tool for investigating the physiological relationships between the d-RCDN and examined muscles. For pharmacological studies on WWamide-1, we employed the ABRM of *M. edulis* [1] and radula protractor muscle of *R. thomasiana* [10–12] because their pharmacological backgrounds have been investigated in detail. At concentrations of 10^{-8} to 10^{-6} M, WWamide-1 displayed a dose-dependent inhibition on the phasic contractions of ABRM of *M. edulis* (Fig. 4A). However, the peptide at 10^{-6} M did not affect contractions evoked by either 10^{-5} M ACh or 10^{-6} M FMRFamide (Fig. 4B,C). Since ACh and FMRFamide are thought to act on the post-synaptic sites of ABRM [1], these results suggest that the peptide acts pre-synaptically to inhibit neuromuscular transmission. WWamide-1 exhibited marked excitatory effects on the radula protractor muscle of *R. thomasiana*. Although both ACh and Glu evoke muscular contractions by acting directly on muscle fibres, the principal excitatory neurotransmitter has been suggested to be ACh [11]. The peptide potentiated muscle twitches at 10^{-9} M or higher and caused repetitive vigorous contractions after each twitch at 10^{-7} M in the low-magnesium (20 mM $MgCl_2$) ASW (Fig. 5A_1).

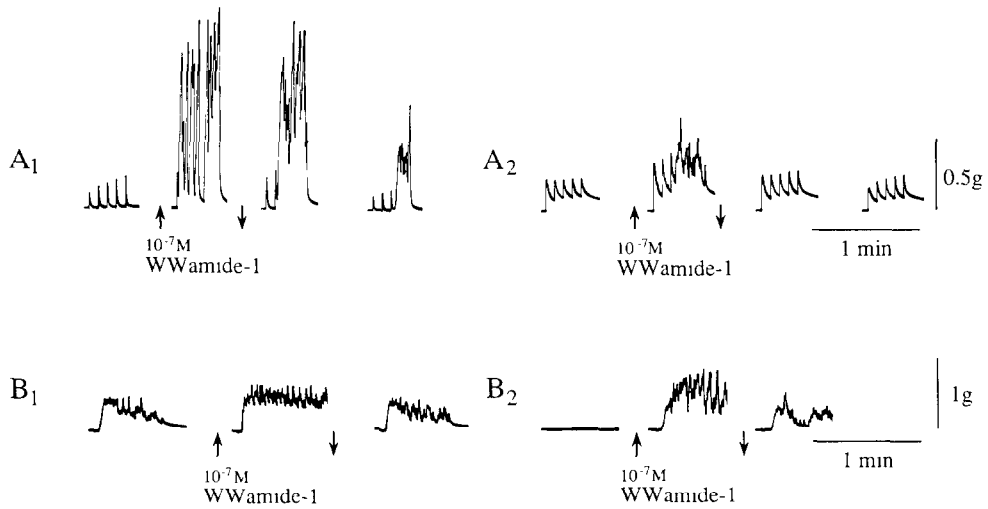

Fig. 5. Excitatory activities of WWamide-1 on the radula protractor muscle of *R. thomasiana*. (A_1) Effect of 10^{-7} M WWamide-1 on twitch contractions (15 V, 2 ms, 0.2 Hz, 5 pulses) in low-magnesium (20 mM $MgCl_2$) ASW. (A_2) Effect of 10^{-7} M WWamide-1 on twitch contractions (15 V, 5 ms, 0.2 Hz, 5 pulses) in high-magnesium (100 mM $MgCl_2$) ASW. (B_1) Effect of 10^{-7} M WWamide-1 on contractions induced by 10^{-6} M ACh in low-magnesium (20 mM $MgCl_2$) ASW. (B_2) Effect of 10^{-7} M WWamide-1 on contractile effects of 10^{-4} M Glu in low-magnesium (20 mM $MgCl_2$) ASW. (Note: Glu induced contractions at 10^{-3} M in the preparation.)

To investigate if the effect was attributed to either pre- or post-synaptic action, the following experiments were performed. The electrical pulses (15 V, 2 ms, 0.5 Hz, 5 pulses) used in Fig. $5A_1$ are considered to cause contractions by stimulating both the nerve and muscle fibres [10]. When the concentration of $MgCl_2$ was increased to 100 mM, magnesium ions blocked the neuromuscular transmission, and stimuli of longer duration pulses (5 ms) elicited muscular contractions (Fig. $5A_2$) probably by direct stimulations of the muscle fibres [12]. Under such conditions, 10^{-7} M WWamide-1 potentiated the contractions although the magnitude of potentiation was smaller than that in Fig. $5A_1$. This result suggests that the peptide acts on the post-synaptic membrane to potentiate contractions of the muscle. The post-synaptic action of the peptide was further supported by the effects on ACh- and Glu-induced contractions. The peptide potentiated ACh-induced contractions of the muscle (Fig. $5B_1$) at 10^{-7} M. Although 10^{-4} M Glu per se did not produce any contractions in this muscle without the peptide, marked contractile responses to a similar Glu concentration were elicited in the presence of the peptide (Fig. $5B_2$).

WWamide-1 demonstrated inhibitory activities on the d-RCDN of *A. fulica*. In the peripheral nervous systems of the snail and other molluscs, the effects were modulatory; either inhibitory or potentiating on the muscular contractions. Since WWamide-1 displayed such activities on several molluscan muscles, WWamides and/or related peptides might be distributed extensively in molluscs and play roles in controlling muscular contractions.

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