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APGW-AMIDE AS AN INHIBITORY NEUROTRANSMITTER OF ACHATINA FULICA FERUSSAC

Guo Jun Liu[1], Divina E. Santos[1], Hiroshi Takeuchi[1,*], Yoshimi Kamatani[2], Hiroyuki Minakata[2], Kyosuke Nomoto[2], Ichiro Kubota[3], Tetsuya Ikeda[4] and Yojiro Muneoka[4]

[1]Department of Physiology, Gifu University School of Medicine, Gifu 500, Japan

[2]Suntory Institute for Bioorganic Research, Osaka 618, Japan

[3]Suntory Bio-Pharma Tech Center, Gunma 370-05, Japan

[4]Physiological Laboratory, Faculty of Integrated Arts and Sciences, Hiroshima University, Hiroshima 730, Japan

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SUMMARY: APGWamide (L-Ala-L-Pro-Gly-L-Trp-NH_2) was purified from the ganglia of an African giant snail (Achatina fulica Ferussac). This peptide inhibited (hyperpolarized) more than half of the Achatina neurone types tested. This produced an outward current with the membrane conductance increase of RAPN (right anterior pallial neurone) under voltage clamp. The ED_{50} of the peptide was 6.2×10^{-8} M (95 % confidence limit: 5.0 - 7.8 x 10^{-8} M) and the E_{max} was 3.9 ± 0.2 nA. The effects were due to a membrane permeability increase to K^+. The peptide is proposed as an inhibitory neurotransmitter of the Achatina neurones.

APGWamide (L-Ala-L-Pro-Gly-L-Trp-NH_2), a tetrapeptide closely related to the C-terminal of the crustacean red-pigment concentrating hormone (RPCH), was recently isolated from the ganglia of a prosobranch, Fusinus ferrugineus. This peptide potentiated the twitch contraction of the radula retractor muscle of the same animal, and inhibited the tetanic contraction of the anterior byssus retractor muscle (ABRM) of Mytilus edulis[1]. The present study aimed to examine the possible role of the peptide as a neurotransmitter of Achatina fulica neurones: its presence in the Achatina ganglia; the mapping of the Achatina neurone types sensitive to this peptide in comparison with FMRFamide[2] and Ser^2-Mytilus inhibitory peptide (Ser^2-MIP)[3]; the measurement of the dose-response curve and the analysis of the ionic mechanism of its effects.

*To whom correspondence should be addressed.

ABBREVIATIONS: HPLC, the high performance liquid chromatography. SIMS, secondary ionization mass spectrometry. ED_{50}, effective dose 50. E_{max}, maximal effects. E_K, equilibrium potential of potassium.

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MATERIALS AND METHODS

Animals: An African giant snail (Achatina fulica Ferussac) was collected in Manila, Philippines. The living snail for the pharmacological study, and the suboesophageal and cerebral ganglia dissected in the frozen state for the peptide isolation, were transported to Japan by air. A mussel (Mytilus edulis) was collected in Hiroshima Bay, Japan.

Purification: The peptide was purified from the Achatina ganglia by a HPLC system similar to that previously used to isolate MIP from the Mytilus ganglia[3]. After each purification step, the fractions were bioassayed with contraction of the Mytilus ABRM. The ganglia (59 g in wet weight) from 940 specimens of Achatina were homogenized in 80 % acetone at 0 - 4 °C for three times. After centrifugation (16,000 g, 40 min), the supernatant was evaporated in vacuo into about 1 ml, dissolved in 0.1 N HCl, and again centrifuged (16,000 g, 40 min). The supernatant was passed through Sep-Pak C_{18} cartridges. The retrained material was eluted by 100 % ethanol and applied to a column of Asahi Pak ODP-50. The column was eluted with a linear gradient of 0 - 60 % acetonitrile in 0.1 % TFA (pH 2.2) at a flow rate of 1.0 ml/min. The active fractions were divided into four groups. APGWamide was obtained from the group 1 which was consisted of the fractions No. 21 - 23. The active material was subjected to a column of TSK gel SP-5PW and eluted with a 60 min gradient of 0 - 0.6 M NaCl in 10 mM phosphate buffer (pH 6.9) at a flow rate of 0.5 ml/min. The active fractions, No. 17 - 20, were applied to a column of TSK gel ODS-80TM and eluted with a 50 min gradient of 10 - 20 % acetonitrile in 0.1 % TFA. The active fractions were again applied to the ODS-80TM and eluted with 12.5 % acetonitrile in 0.1 % TFA (Fig. 1, A, a). The active fraction was further applied to the same column and eluted with 12.0 % acetonitrile (Fig. 1, A, b).

Structure determination and peptide synthesis: After hydrolysed in 6 N HCl, the amino acid composition of the purified peptide was determined (Tosoh CCP-8000). The amino acid sequence of the native peptide was analyzed by the automated Edman degradation with a gas-phase sequencer (Shimadzu PSQ-1). The molecular weight of the peptide was determined by SIMS (Hitachi M-80B). The authentic APGWamide was synthesized by the conventional solid-phase method.

Pharmacological study: The dissected Achatina ganglia were incubated with 0.67 % trypsin (type III, obtained from Sigma Chemical) for 10 min, and the connective tissue covering the ganglia were removed. The mapping study of the neurone types sensitive to the peptide was performed with the sixteen neurone types under current clamp (Table 1)[4-7]. The further pharmacological study was carried out with RAPN (right anterior pallial nerve neurone) having a resting membrane potential of about -50 mV, under the conventional voltage clamp using the two microelectrodes[8]. The holding voltage (V_h) was maintained mainly at -50 mV. FMRFamide was obtained from Peptide Institute, Japan, and Ser-MIP was synthesized by our laboratories. The Achatina physiological solution has been formulated from the inorganic ion composition in its hemolymph[9] as follows: NaCl (65.6 mM), KCl (3.3), $CaCl_2$ (10.7), $MgCl_2$ (13.0), Tris HCl (9.0) and Tris base (1.0). The peptides were applied either by pneumatic pressure ejection (2 x 10^5 Pa, 400 msec and 10^{-3} M) or by bath in the experimental chamber of 0.2 ml. The dose-response curve of the peptide was analyzed by the probit method[10] using a computer program.

RESULTS

APGWamide occurrence: The amino acid composition of the native peptide was Ala, 1.0; Pro, 0.99; Gly, 0.93. The amino acid sequence was determined to be Ala-Pro-Gly-Trp, and the molecular weight determined by SIMS [$(M+H)^+$ m/z 430] was 429, indicating this peptide to be APGWamide. The behavior on HPLC and the effects on the ABRM of Mytilus of the synthetic APGWamide were identical to the native one (Fig. 1, A, c and d). The isolation yield of the peptide was 35 nM from 940 animals.

APGWamide-sensitive neurones: Of the sixteen neurone types tested, the ten were hyperpolarized by the pneumatic pressure ejection of the synthetic

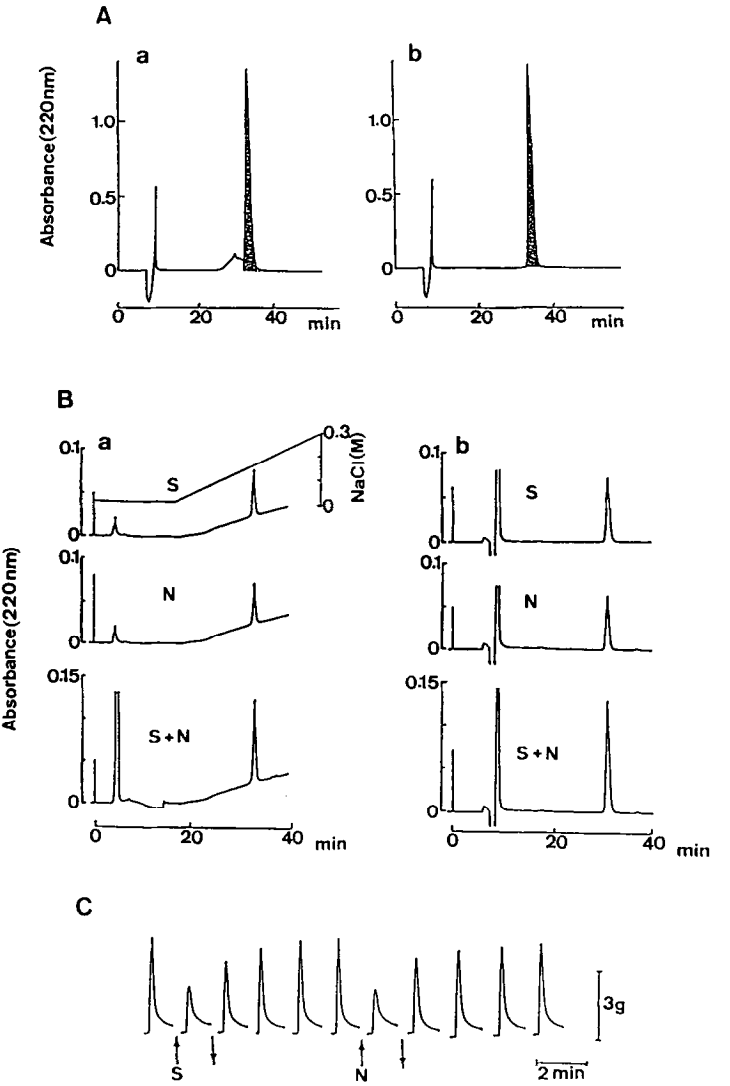

FIGURE 1. Purification of APGWamide from Achatina ganglia. A, Chromatograms at the third (a) and the fourth (b) steps of the HPLC purification. HPLC column and its conditions are given in the text. In a and b, the active peaks are shown by dotting. B, HPLC profiles of the synthetic (S) and native (N) peptides and their mixture (S + N). In a, the cation-exchange column (TSK SP-5PW) was eluted with a 30 min linear gradient of 0 - 0.3 M NaCl in 10 mM phosphate buffer at pH 6.9. In b, the reversed-phase column (TSK gel ODP-80TM) was eluted isocratically with 12.5 % acetonitrile in 0.1 % TFA at pH 2.2. C, inhibitory effects of synthetic (S) and native (N) peptides on the phasic contractions of the Mytilus ABRM produced by the repetitive electrical stimulation (15 V, 3 msec, 19 Hz for 5 sec) applied at 10 min intervals. The peptides (S and N) were introduced at the upward arrow (↑) and washed out at the downward arrow (↓). S, 3 x 10^{-7} M; N, from ganglia of 10 animals.

APGWamide under current clamp (Fig. 2, A, a). FMRFamide and Ser^2-MIP also showed hyperpolarizing effects on the majority of Achatina neurone types (Table 1). None was depolarized by the three peptides mentioned.

Outward current: The RAPN neuromembrane produced an outward current (I_{out}) accompanied with the membrane conductance increase (Δg) by the pneu-

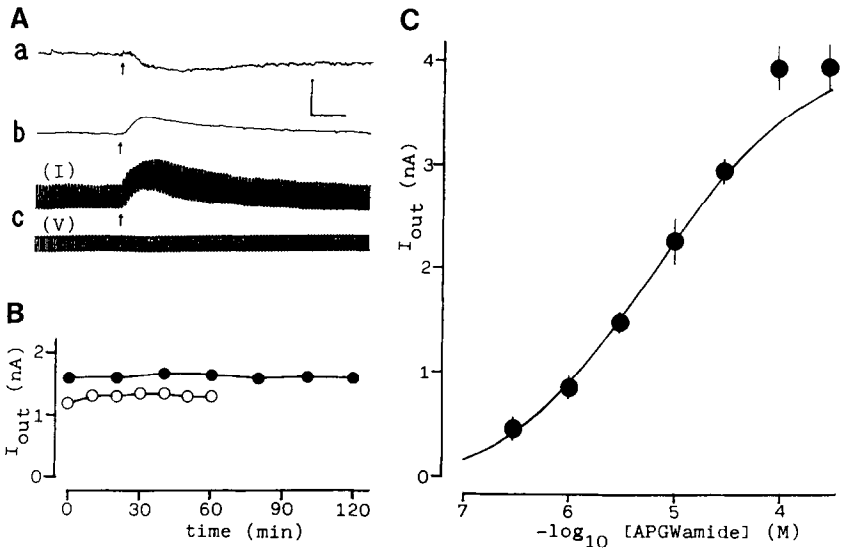

FIGURE 2. Inhibitory effects of APGWamide on Achatina RAPN (right anterior pallial nerve neurone). A, the pneumatic pressure ejection of the peptide (2 x 10^{-5} Pa, 400 msec, 10^{-3} M) (arrows). a, hyperpolarization in current clamp (resting membrane potential of the neurone: about -50 mV). b, outward current (I_{out}) in voltage clamp (holding voltage (V_h): -50 mV). c, membrane conductance increase with I_{out}. The repetitive hyperpolarizing pulses (5 mV, 1 sec, 0.5 Hz) were superimposed on V_h. (I), membrane current; (V), membrane voltage (5 mV). Vertical bar, calibration (20 mV for a, 2 nA for b and c (I), and 10 mV for c (V). Horizontal bar, time course (20 sec). B, response stability to the repetitive applications of the peptide. Abscissa, time course (min). Ordinate, I_{out} produced by the peptide (nA). (●), bath application at 10^{-5} M for 1 - 1.5 min; (○) pressure ejection. C, dose-response curve for producing I_{out} (n = 4). The peptide was applied by bath for 1 - 1.5 min from the lowest concentration with the interval of washing for 20 min. Abscissa, peptide concentrations in -log scale in M. Ordinate, I_{out} (nA) (small bar: S.E.M.). The smooth line was drawn by fitting to the ideal sigmoidal curve calculated by the computer.

matic pressure ejection of APGWamide(M ± S.E.M.); I_{out} was 1.38 ± 0.11 nA, and Δg was 0.07 ± 0.01 μS (n = 4). In the Ca^{2+}-free and Mg^{2+}-rich (3 x) medium, to avoid possibility of the transsynaptic influences, the responses to the peptide were similar, suggesting that the peptide acted directly on the neurone tested (Fig. 2, A, b & c). The RAPN responses to APGWamide were

TABLE 1. Achatina neurone types sensitive to APGWamide, FMRFamide and Ser^2-MIP, applied by the pneumatic pressure

No.	Neurone	APGWa	FMRFa	S-MIP*	No.	Neurone	APGWa	FMRFa	S-MIP*
				I. Suboesophageal ganglia					
1.	PON	(-)	(-)	(-)	7.	INN	I	I	I
2.	TAN	I	I	I	8.	VIN	(-)	(-)	(-)
3.	TAN-3	I	I	I	9.	d-VLN	I	(-)	I
4.	RAPN	I	I	I	10.	d-LPeCN	I	I	I
5.	BAPN	I	I	I	11.	LPeNLN	(-)	I	I
6.	d-RPLN	I	(-)	I	12.	d-RPeAN	(-)	I	I
				II. Cerebral ganglia					
13.	d-LCDN	(-)	I	I	15.	v-LCDN	I	I	I
14.	d-RCDN	(-)	I	I	16.	v-RCDN	I	I	I

I, inhibitory effects. (-), no effect.
*, previously reported[11, 12].

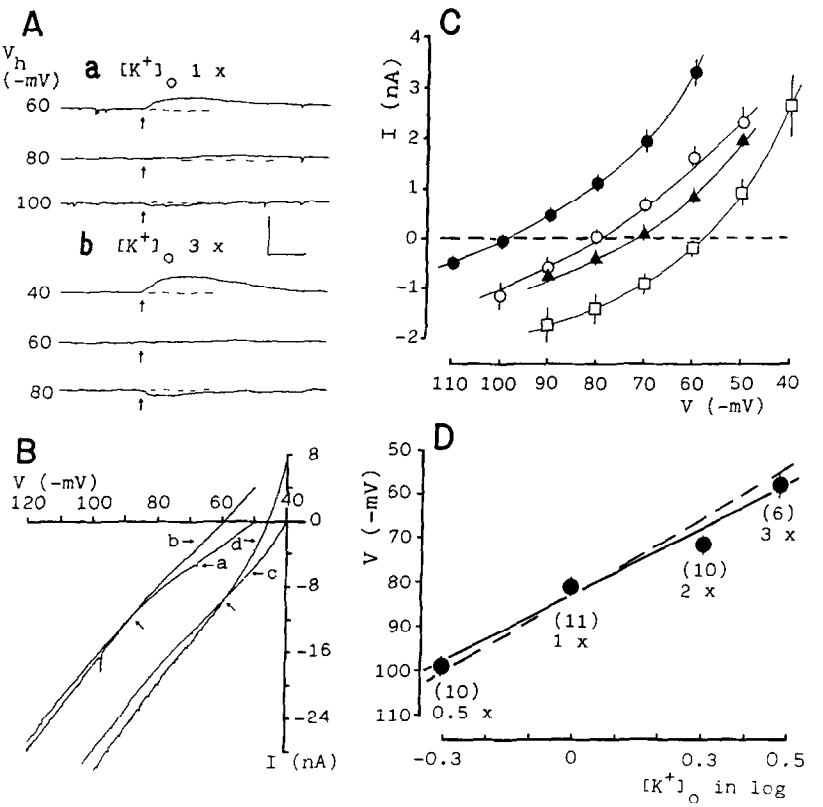

FIGURE 3. Equilibrium potential of APGWamide (E_{APGWa}) on RAPN. A, pneumatic pressure ejection of the peptide in different V_h (arrows). a, normal extracellular K^+ concentration ($[K^+]_o$) (1 x, 3.3 mM). b, high $[K^+]_o$ (3 x, 9.9 mM). Vertical bar, calibration (4 nA); horizontal bar, time course (20 sec). B, bath application of the peptide at 3×10^{-5} M with respect to I-V curves. Abscissa, membrane voltage (V, -mV). Ordinate, membrane current (I, nA). I-V curves in absence (a) and presence (b) of the peptide were measured under normal $[K^+]_o$; those in its absence (c) and presence (d) under the high $[K^+]_o$ (3 x). Two arrows without letter indicate E_{APGWa} values in the normal $[K^+]_o$ (left, -87.5 mV) and the high $[K^+]_o$ (right, -58.5 mV). C, currents produced by the peptide (I_{APGWa}) at 3×10^{-5} M in function of membrane potential (from I-V curve measurements as shown in B). Abscissa, membrane voltage (V, -mV). Ordinate, I_{APGWa} (I, nA) (small bar: S.E.M.). (●), $[K^+]_o$ = 0.5 x, 1.65 mM (n = 10); (○), $[K^+]_o$ = 1 x (n = 11); (▲), $[K^+]_o$ = 2 x, 6.6 mM (n = 10); and (□), $[K^+]_o$ = 3 x (n = 6). Smooth curves were drawn by eyes. The cross points of the curves and the dotted line show E_{APGWa}. D, E_{APGWa} in different $[K^+]_o$. Abscissa, relative $[K^+]_o$ in log scale. Ordinate, E_{APGWa} (V, -mV) (small bar: S.E.M.). Smooth line was drawn by fitting to linear regression of the measured E_{APGWa}, and the dotted line to Nernst equation for E_K through E_{APGWa} in the normal $[K^+]_o$. Number of trials are shown in parenthesis.

stable, when applied by bath at 10^{-5} M for 1 - 1.5 min with the interval of washing for 20 min, or by the pressure ejection with the interval for 10 min (Fig. 2, B).

Dose-response relations: The dose-response curve of APGWamide, applied by bath, for producing the RAPN I_{out} was analyzed by the probit method (n = 4): ED_{50} was 6.2×10^{-6} M (confidence limit at 95 %: 5.0 - 7.8×10^{-6} M), the tangent slope of the curve at ED_{50} was 0.40, the coefficient for the ideal sigmoidal curve was 0.999, the apparent Hill coefficient was 0.67 (r = 0.999), and E_{max} was 3.93 ± 0.21 nA (Fig. 2, C).

Ionic mechanism: The equilibrium potentials of the APGWamide-induced currents (E_{APGWa}) in RAPN, measured by the pneumatic pressure ejection on the different V_h, were -80 - -90 mV in the normal potassium concentration ($[K^+]_o$) (1 x, 3.3 mM), and about -60 mV in the high $[K^+]_o$ (3 x, 9.9 mM) (Fig. 3, A). E_{APGWa}, measured by recording the current-voltage curves (I-V curves) in absence and presence (by bath) of APGWamide at 3×10^{-5} M (the cross point of the two curves shows E_{APGWa}) were (M ± S.E.M.): -98.85 ± 1.11 mV for $[K^+]_o$ 0.5 x (1.65 mM), -80.89 ± 1.56 mV for normal $[K^+]_o$, -72.00 ± 0.97 mV for $[K^+]_o$ 2 x (6.6 mM), and -57.75 ± 1.20 mV for $[K^+]_o$ 3 x, which were comparable to those by the pressure ejection (Fig. 3, B & C). The relation between E_{APGWa} (Y, in mV) and relative $[K^+]_o$ (X) is obtained by the linear regression: $Y = -83.3061 + 49.6326\ X$ ($r = 0.986983$), which is quite linear. The same relation according to Nernst equation (at 20 .C) for E_K through E_{APGWa} value in normal $[K^+]_o$ is: $Y = -80.89 + 58.11\ X$. The latter equation is comparable to the former, indicating that the inhibitory effects of APGWamide are, at least mainly, due to the membrane permeability increase to K^+ (Fig. 3, D).

DISCUSSION

The presence of APGWamide in the ganglia and its inhibitory effects on the giant neurones of Achatina fulica were demonstrated in the present study. This peptide, as well as FMRFamide and Ser^2-MIP, inhibited the majority of the Achatina neurone types tested, and excited none of them. The maps of the neurone types sensitive to each of the three inhibitory peptides were not largely different[11,12]. The ionic mechanism for APGWamide effects on RAPN was an increase in K^+ permeability, which is identical to that for Ser^2-MIP on the same neurone type[11]. In contrast to the inhibitory peptides, achatin-I, which has been isolated from the Achatina ganglia, excited about half of the Achatina neurones tested, and inhibited none of them[13,14].

On the other hand, each of the putative neurotransmitters of amines and amino acids, such as dopamine, 5-hydroxytryptamine, acetylcholine and GABA, showed both excitatory and inhibitory effects according to the neurone types tested[4-7]. This indicates that the combinations of receptors and ionic channels to be activated by these peptides are less multiple than those activated by amines and amino acids.

The dose-response curve of APGWamide was sigmoidal, likewise those of Ser^2-MIP and achatin-I. ED_{50} and E_{max} values of the latter two peptide on the Achatina neurones were: 3×10^{-5} M and 10 nA for Ser^2-MIP on RAPN and $0.2 - 2.7 \times 10^{-5}$ M and 4.2 - 6.3 nA for achatin-I on several neurone types. The ED_{50} of APGWamide was lower than those of the two other peptide, but its E_{max} was approximately similar to achatin-I and less than Ser^2-MIP[11,14]. The Hill coefficient of APGWamide deviated from 1.0 like achatin-I[14], suggesting that the peptide may produce some allosteric changes of their receptors.

Following our previous proposal for achatin-I as an excitatory neurotransmitter of Achatina neurones[13,14], we propose APGWamide as an inhibitory transmitter of these neurones based on its presence in native animals and marked effects. Although FMRFamide and Ser^2-MIP also show inhibitory effects, these may be the potent agonists of the transmitters, since their presence in the Achatina ganglia is not yet demonstrated.

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