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A MOLLUSCAN NEUROPEPTIDE RELATED TO THE CRUSTACEAN HORMONE, RPCH

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A peptide that potentiates twitch contraction of the radula retractor muscle of the prosobranch mollusc Fusinus ferrugineus was isolated from the ganglia of the animal. Its primary structure is H-Ala-Pro-Gly-Trp-NH_2 (APGWamide) closely related to the C-terminal tetrapeptide of the crustacean red-pigment-concentrating hormone. APGWamide showed modulatory actions on contractions in various molluscan muscles.

A number of neuropeptides which belong to the family of RPCH-AKH have been isolated from arthropods, but none of such peptides have been found in other phyla[1]. However, Greenberg et al. have strongly suggested that molluscan ganglia possess a peptidic substance related to RPCH-AKH[2]. In the present study, we isolated a RPCH-related bioactive tetrapeptide from the ganglia of a prosobranch mollusc, Fusinus ferrugineus. We report here

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Abbreviations: ABRM, anterior byssus retractor muscle; ACh, acetylcholine; AKH, locust adipokinetic hormone; APGWamide, H-Ala-Pro-Gly-Trp-NH_2; ASW, artificial seawater; FAB-MS, fast atom bombardment mass spectrometry; Hepes, N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid); HPLC, high performance liquid chromatography; 5-HT, serotonin; PTH, phenylthiohydantoin; RPCH, crustacean red-pigment-concentrating hormone; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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the purification, structure determination and biological activities of the RPCH-related molluscan neuropeptide.

MATERIALS AND METHODS

Animals---The animals used in the experiments are as follows: Fusinus ferrugineus (Prosobranchia), Euhadra congenita (Pulmonata) and Mytilus edulis (Bivalvia). Euhadra were collected in the campus of Hiroshima University. Fusinus and Mytilus were collected from Hiroshima Bay.

Purification---The cerebral, suboesophageal and buccal ganglia were excised from 1,100 of Fusinus. The acetone extract of the ganglia was forced through C-18 cartridges (Waters Sep-Pak). The retained material was eluted with methanol and gel-filtrated with a column of Sephadex G-15 (26x400 mm). These procedures were the same as those described previously[3]. The fractions showed four peaks of activities on twitch contractions of the radula retractor of Fusinus. However, each of the peaks seemed to be eluted partially overlapping with one or two other peaks. Therefore, we divided the active fractions into two groups, fractions 21-40 and fractions 41-58. The RPCH-related peptide was obtained from the latter group.

The fractions of the latter group were pooled, concentrated and subjected to HPLC (Jasco TRI ROTAR VI) separation with a C-18 reversed-phase column (Tosoh ODS-80TM). The column was eluted with a 60-min linear gradient of 0-60% acetonitrile in 0.1% TFA at pH 2.2. Three peaks of activities were obtained, twitch-potentiating, contractile and twitch-inhibiting peak. The active substance in the potentiating peak was then applied to a cation-exchange column (Tosoh SP-5PW) and eluted with a 50-min linear gradient of 0-0.5 M NaCl in 10 mM phosphate buffer at pH 6.8 (Fig. 1A). Final purification was performed by applying the active material again to the C-18 reversed-phase column and eluting with a 30-min linear gradient of 15-20% acetonitrile in 0.1% TFA at pH 2.2 (Fig. 1B).

After each purification step, the bioactivity of each fraction was examined on train of twitch contractions of the isolated Fusinus radula retractor mounted in a chamber (2 ml). The methods of dissection, stimulation and recording from the muscle were the same as those described previously[3]. The potentiating activity of the purified substance on the twitch contractions is shown in Fig. 1C.

Structure determination---The purified active substance was subjected to amino acid sequence analysis by automated Edman degradation with a gas-phase sequencer (Applied Biosystems 477A) coupled with a PTH-amino acid analyzer (Applied Biosystems 120A), amino acid analysis (Hitachi L-8500) and FAB-MS analysis (JEOL JMS HX-100), and thus the probable structure of the substance was determined to be a tetrapeptide amide, APGWamide.

The peptide was then synthesized by a manual solid-phase method followed by HF cleavage and HPLC purification. Its behavior on HPLC and bioactivity on the radula retractor were compared with those of the native peptide.

Biological actions---Biological actions of APGWamide were examined on the following molluscan muscles: the radula retractor and protractor of Fusinus, the crop and the pharyngeal retractor of Euhadra, and the ABRM and the pedal retractor of Mytilus. These muscles, except the crop, were isolated and mounted in the same chamber used in the bioassay experiments. The methods of stimulation of the muscles and recording of tension from them

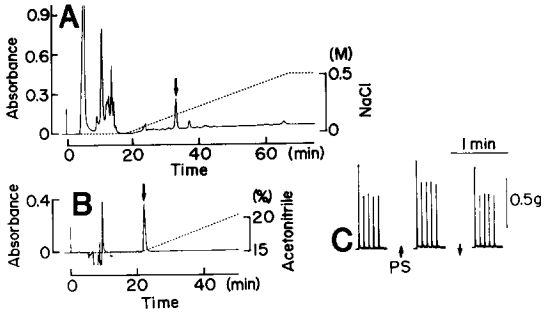

Fig. 1 HPLC purification of twitch-potentiating substance from the ganglia of Fusinus and bioactivity of the purified substance. A: the second step of the purification with a cation-exchange column. B: The third (final) step of the purification with a C-18 reversed-phase column. In A and B, bioactive peaks were coincident with the absorbance peaks indicated by arrows. C: potentiating action of the purified substance on train of twitches of the radula retractor of Fusinus. The train contractions were evoked by applying repetitive electrical pulses of stimulation (15 V, 2 msec, 0.2 Hz, 5 pulses) at 10 min intervals. The purified substance (PS) was applied to the muscle 8 min prior to the stimulation and washed out soon after it.

were also the same as those used in the bioassay experiments. APGWamide was applied to the muscles by replacing the solution in the chamber with the solution containing the peptide. The crop was excised by cutting at its both ends. The surrounding tissues were removed. The preparation was suspended in a 2-ml aerated organ bath. APGWamide was applied to the muscle by injecting the stock solution into the bath.

Salines---The physiological saline for Mytilus muscles was ASW of the following composition: 445 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 55 mM $MgCl_2$ and 10 mM Tris-HCl; pH 7.8. For the saline of the Fusinus muscles, low-Mg^{2+} ASW (20 mM $MgCl_2$ ASW) was used to obtain large twitch contractions. This saline was made by replacing a part of $MgCl_2$ in the normal ASW with osmotically equivalent NaCl. The physiological saline for the muscles of the land snail Euhadra was of the following composition: 120 mM NaCl, 4mM KCl, 0.9 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1.5 mM glucose and 10 mM Hepes-NaOH; pH 7.5.

RESULTS AND DISCUSSION

The determined sequence and detected amount (pmol) of each amino acid in the amino acid sequence analysis of the purified substance were as follows: $Ala_{65.3}$-$Pro_{60.1}$-$Gly_{59.3}$-$Trp_{33.5}$. Quantitative amino acid analysis showed the following amino acid composition normalizing on Ala=1: Pro, 0.8; Gly, 1.0; Ala, 1.0. From these results, the substance is assumed to be a peptide whose structure is H-Ala-Pro-Gly-Trp-OH or its C-terminal amide

derivative. A molecular ion peak of 429.1 m/z $(M+H)^+$ in the FAB-MS spectrum of the substance was in agreement with an amide at the C-terminus.

The synthesized APGWamide and the native peptide were eluted at the identical positions when applied to the C-18 reversed-phase column and the cation-exchange column. A mixture of the peptides showed a single absorbance peak in both of the cases of elution (Fig. 2A and 2B). The relationship between dose and twitch-potentiating action in the Fusinus radula retractor for both peptides were almost identical (Fig. 2C). Thus, we concluded that the primary structure of the Fusinus peptide is H-Ala-Pro-Gly-Trp-NH_2 (APGWamide), which is related to the C-terminal tetrapeptide of RPCH (pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2). The total yield of APGWamide calculated from the result of the amino acid analysis was 3.01 nmol from 1,100 animals.

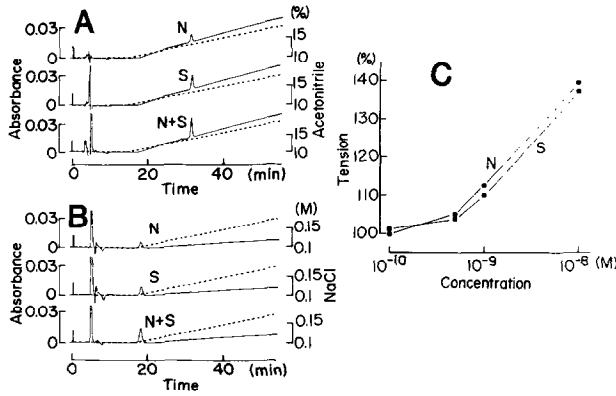

Fig. 2 Comparison of chromatographic properties and bioactivity of native peptide with those of synthetic APGWamide. A: reversed-phase chromatograms of the native (N) and synthetic (S) peptides and their mixture (N+S). B: cation-exchange chromatograms of the native (N) and synthetic (S) peptides and their mixture (N+S). C: potentiating activities of the native (N) and synthetic (S) peptides on total tension of 5 train twitches of the radula retractor of Fusinus. Each total tension is indicated as a percentage of the control total tension. The procedures for application of the peptides and recording of train twitches were the same as in Fig. 1C. The concentrations of the native peptide were calculated from the result of amino acid analysis.

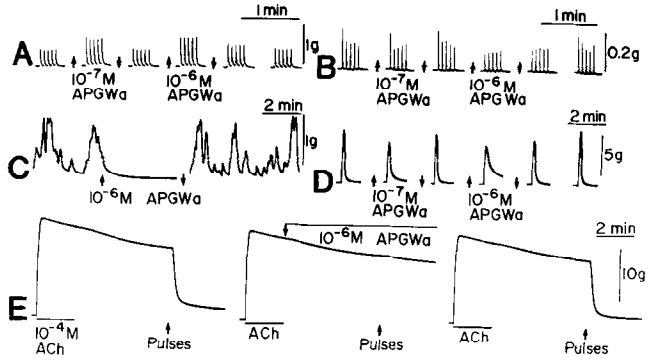

Fig. 3 Effects of APGWamide on some molluscan muscles. A: potentiating effect on train twitches of the radula retractor of Fusinus. B: inhibitory effect on train twitches of the radula protractor of Fusinus. The procedures in A and B were the same as in Fig. 1C. C: Inhibitory effect on spontaneous contractions of the crop of Euhadra. At downward arrow, the crop was washed with normal saline for 10 min. D: inhibitory effect on tetanic contraction of the ABRM of Mytilus. Tetanic contraction was evoked by applying repetitive electrical pulses of stimulation (15 V, 3 msec, 10 Hz, for 5 sec) at 10 min intervals. The other procedures were the same as in Fig. 1C. E: inhibitory effect on relaxation of catch tention in response to repetitive electrical pulses of stimulation (15 V, 2 msec, 1 Hz, 15 pulses) in the ABRM of Mytilus. Catch tension was produced by applying ACh for 2 min at 20 min intervals.

Although APGWamide showed a potentiating action on twitch contractions in the radula retractor of Fusinus, it showed an inhibitory action in the radula protractor (Fig. 3A and B). The thresholds for the potentiation and inhibition were approximately $5x10^{-10}$ M and $5x10^{-8}$ M, respectively. In contrast to the twitches, ACh contractions in both of the muscles were not affected even by 10^{-5} M of the peptide.

Spontaneous contractions of the crop (Fig. 3C) and twitch contraction of the pharyngeal retractor of Euhadra were inhibited by APGWamide. Tetanic contractions of the ABRM (Fig. 3D) and the pedal retractor of Mytilus were also inhibited by the peptide. In these muscles, the thresholds for the inhibition were between $3x10^{-10}$-10^{-9} M. ACh contraction of the ABRM was not inhibited but slightly potentiated by 10^{-7} M or higher APGWamide.

In the tetanic contraction of the ABRM, APGWamide was found to inhibit not only tension development but also relaxation (Fig.

3D). Therefore, we examined the effect of APGWamide on relaxation of catch tension in response to various relaxing stimuli. The peptide showed potent inhibitory effect on the relaxation in response to repetitive electrical pulses of stimulation (Fig. 3E), but it showed no effect on the relaxation in response to relaxing monoamines[4], such as 5-HT, and catch-relaxing peptide[5]. The threshold for the inhibition of the relaxation by repeititve electrical stimulation was approximately $5x10^{-10}$ M.

It has been suggested that repetitive electrical pulses applied to the ABRM relax catch by stimulating the intramuscular relaxing nerves to release relaxing transmitter 5-HT[6,7]. Therefore, the results obtained in the experiments of the ABRM suggest that APGWamide inhibits release of 5-HT from the relaxing nerve terminals by acting on the intramuscular nerve elements. RPCH has also been suggested to inhibit release of the relaxing transmitter in the ABRM[8]. It has been shown that 5-HT potentiates tetanic contraction of the ABRM[7,9]. The decrease in peak tension of the tetanic contraction in APGWamide might be a result of inhibitory action of the peptide on 5-HT release. We tested 5-HT on the radula muscles of Fusinus and found that the twitch contractions of the retractor were inhibited by the amine, whereas those of the protractor were potentiated. The actions of APGWamide on the twitch contractions of the radula muscles might be results of inhibition of 5-HT release. Yanagawa et al. have shown that RPCH potentiates twitch contractions of the radula retractor of Rapana thomasiana which is a prosobranch mollusc closely related to Fusinus[10]. In this muscle, 5-HT is also a potent twitch-inhibiting agent[11].

In summary, APGWamide is structurally related to RPCH. In molluscan muscles, further, both of the peptides seem to show

qualitatively similar actions. Some of the actions of the peptides on the muscles may be brought about through inhibition of 5-HT release.

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