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Isolation and Identification of a Novel Ala-Pro-Gly-Trp-amide-Related Peptide Inhibiting the Motility of the Mature Oviduct in the Cuttlefish, *Sepia officinalis*

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HENRY J., P. FAVREL AND E. BOUCAUD-CAMOU. *Isolation and identification of a novel APGW-amide-related peptide inhibiting the motility of the mature oviduct in the cuttlefish, Sepia officinalis.* PEPTIDES **18**(10) 1469–1474, 1997.—A novel myotropic neuropeptide was isolated from 110 optic lobes (OL) of mature females of the cuttlefish *Sepia officinalis* L. by mean of high performance liquid chromatography (HPLC). The peptide inhibits the motility of the oviduct by decreasing the tonus, the frequency and the amplitude of the contractions. The primary structure of the peptide was determined as Gly-Trp-NH_2. This new dipeptide is closely related to the Ala-Pro-Gly-Trp-NH_2 family first identified in gastropod molluscs. On the perfused oviduct, GWa appeared to be 3000 times more potent than APGW-amide. The processing of synthetic APGWa into GWa by diaminopeptidyl activity has been clearly observed in OL extract. Nevertheless, the analysis in MALDI-MS of HPLC OL fractions did not reveal any APGWa related peptides of the known : APGWa, KPGWa, RPGWa and TPGWa. GWa could be processed from a not yet identified APGWa related peptide. © 1997 Elsevier Science Inc.

Sepia officinalis Cephalopods Molluscs Oviduct Neuropeptides APGW-amide

IN both gastropods and cephalopods the different steps associated with reproductive activities appear to be clearly stereotyped. Spermatozoides or spermatophores are deposited by the male(s) into a female receptacle. The fertilization occurs in the female genital tract or in the mantle cavity and finally the egg-mass is bound to a substrate. The behavior as well as the physiological mechanisms associated with reproduction, biosynthesis of yolk proteins and egg-mass formation appear to be very similar both in gastropods and cephalopods. If the peptidergic control of reproduction has been strongly investigated in gastropods such as the freshwater snail *Lymnaea stagnalis* (2,3) and the marine opisthobranch *Aplysia californica* (2,9,10,12,14), in cephalopods the modality of this regulation is quite unknown. The lack of data concerning the endocrinology of this molluscan class is probably related to the difficulty in maintaining live specimens in aquaria and hence performing in vivo experiments. Therefore, in vitro bioassays are an interesting alternative mean to investigate the neuroendocrine control of physiological mechanisms such as egg capsule biosynthesis, vitellogenesis and egg-laying. In the cuttlefish *Sepia officinalis,* the transport of ovocytes is performed by the contractions of a large oviduct opened in the mantle cavity. Therefore, the contractile activity of this organ provides a valuable and reproducible biological test to investigate the peptidergic control of egg-laying and to purify myotropins involved in the ovulation. In this paper, the isolation and the purification were realised from optic lobes using the contraction of the distal part of the oviduct. The optic lobes derive from cerebral ganglia and are involved in the control of egg-capsule biosynthesis in the accessory sex glands (4), one of the events closely related to egg-laying activity.

METHOD

Experimental Animals

All cuttlefish were captured in the western English Channel between January and June by trawling. They were maintained in 250 liters outflow tanks in the Marine Station of Luc-sur-mer (University of Caen, France).

Preparation of the Isolated Oviduct

The oviduct was dissected out from vitellogenic and mature females and suspended from a displacement tranducer (Phymep, Bionic Instruments) connected with a computer managing the recorder and analyser system DATAC (Dispositif d'Acquisition et de Traitement Automatique de la Contraction). The muscle chamber (2.5 ml) was perfused at a flow rate of 0.5 ml.min^{-1} by

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TABLE 1

EXPERIMENTS FOR PROCESSING OF APGWa INTO GWa

Assays	OL Extract	Incubation	APGWa	GWa
1	250μl	1 min 35°C	1μg	no
2	250μl	no incubation	1μg	no
3	250μl	1 min 35°C	1μg	1 μg
4	250μl	no incubation	1μg	1 μg

synthetic filtered sea water (Instant Ocean) containing 1 mM glucose and maintained at 15°C by a cooling system. The high-performance liquid chromatography (HPLC) fractions were injected in the perfusing flow before the cooling system using a three way tap in order to avoid mechanical and thermal stress. The fractions were observed into the muscle chamber by adding phenol red.

Extraction and Sep-Pak Purification

A total of 110 optic lobes from mature females of *Sepia officinalis* were dissected out and stored in liquid nitrogen until extraction. Groups of 10 optic lobes were heated for 3 min at 100°C in bidistilled water (1 g of fresh tissue for 10 ml). The mixture was subsequently sonicated and centrifuged. The supernatants were pooled. The 20 pellets were redissolved in one liter of bidistilled water and the procedure was repeated. The extract was loaded into a C18 Sep-Pak. The Sep-Pak eluates (methanol 100%)

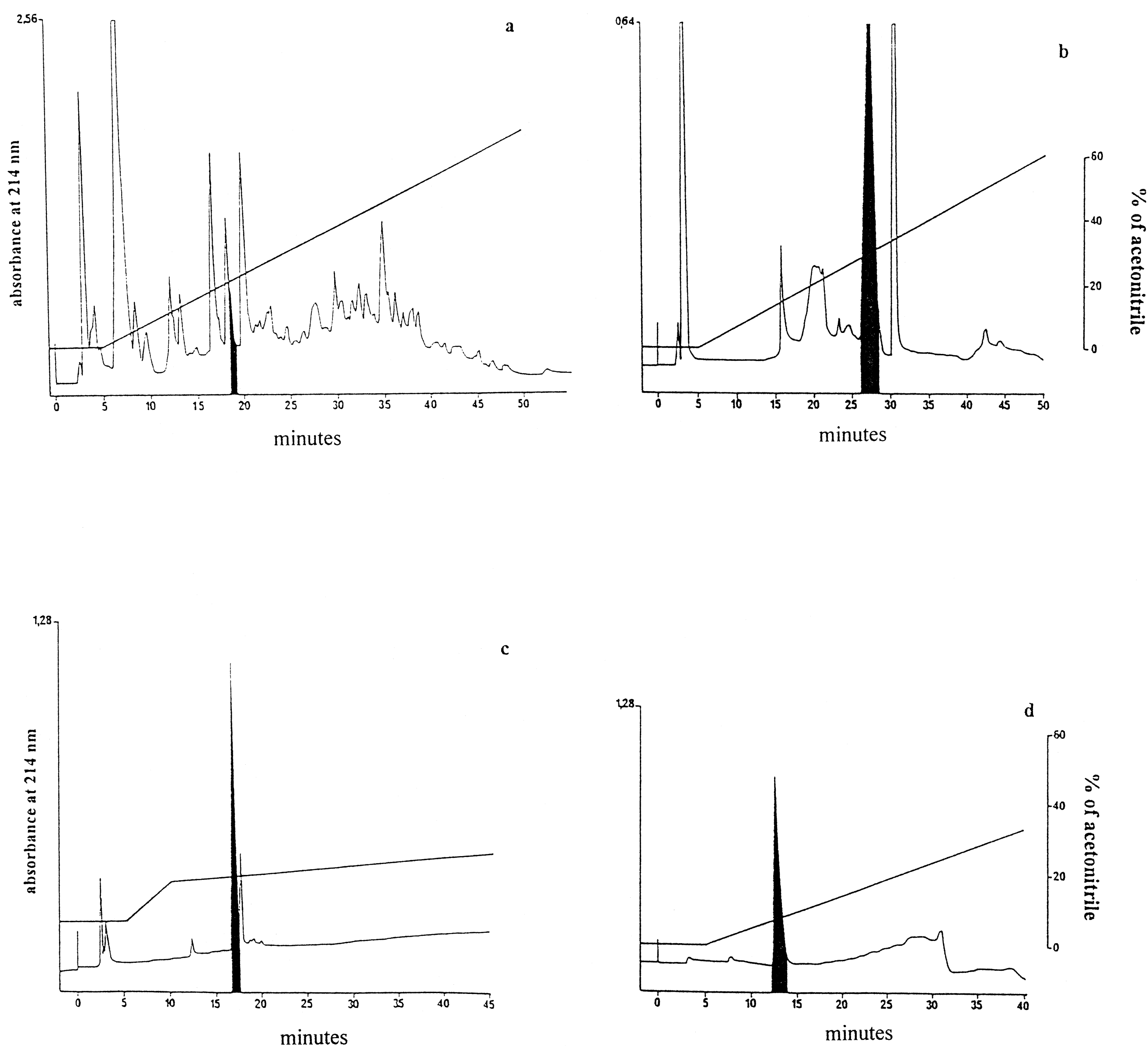

FIG. 1. (a) First step of purification. One of the 20 C18 runs of C18 Sep-Pak eluates of 110 optic lobes. Merck 100RP18 active fraction is eluted between 17.5 and 19 minutes. (b) Second step of purification. Merck 100RP18 active fraction is eluted between 26 and 28 minutes. (c) Third step of purification. Vydac C18 active fraction is eluted between 16 and 17 minutes. (d) Fourth step of purification. Nucleosil phenyl active and pure fraction is eluted between 13 and 14 minutes.

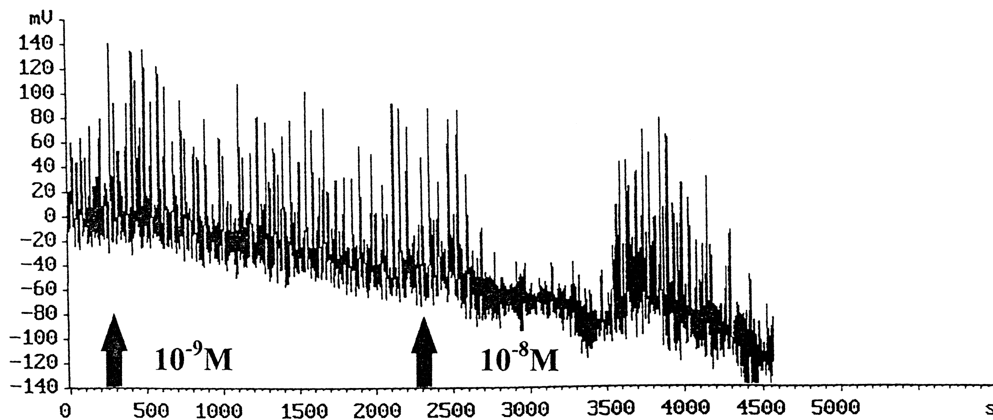

FIG. 2. Decrease of tonus, frequency and amplitude of oviduct contractions following administration of 10^{-8}M of synthetic GWa. For a dose of 10^{-9}M of GWa, the contraction is not affected. The threshold of activity appeared to be between 10^{-8} and 10^{-9}M.

were evaporated in a Speedvac concentrator and kept at −80°C until HPLC fractionation.

High-Performance Liquid Chromatography Purification

HPLC analysis was performed with a D2000 Merck integrator connected with one LKB-2249 HPLC pump and a LKB-2151 variable wave-length detector set at 214 nm. The fractions containing myotropic activity were identified by the oviduct bioassay after each step of purification. For the first separation, the dry eluates were resuspended in 0.1% trifluoroacetic acid (TFA) in water and fractionated using a Merck column 100RP18 (4 × 250 mm, 5 μm) with a linear gradient of 1.33% of acetonitrile (ACN) per minute from 0 to 60% of ACN. One-minute fractions were automatically collected. Ten runs of 10 optic lobes were performed to obtain the first fractionation. The active fractions were resuspended in 25 mM ammonium acetate and injected onto a Merck column 100RP18 (4 × 250 mm, 5 μm) with a linear gradient of 1.33% of ACN per minute from 0 to 60% of ACN. One-minute fractions were automatically collected. The third separation was performed on a Vydac C18 column (3.9 × 250 mm, 5 μm) eluted by two successive gradients of ACN in TFA 0.1% in water: 2.4% of ACN per minute from 0 to 12% of ACN and 0.4% of ACN per minute from 12 to 24% of ACN. The fractions were then manually collected. The last separation was performed on a phenyl Nucleosil column (3.9 × 250 mm, 5 μm) by a linear gradient of 0.7% of ACN per minute from 0% to 40% of ACN in 0.1% TFA in water.

TABLE 2

THE DOSE-DEPENDENT ACTIVITIES

	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M	3.10^{-5}M
APGWa	0	0	0	0	10	20
KPGWa	0	0	0	0	70	/
TPGWa	0	0	0	0	70	/
RPGWa	0	0	0	0	70	/
GWa	0	70	100	100	100	/

The comparative dose-dependent activities of the synthetic peptides expressed in percentage of inhibition of the oviducal contraction.

The pure fraction was manually collected and kept at −20°C until amino acid sequence analysis.

Sequence Analysis

Sequencing of purified peptide was performed using an Applied Biosystems model 473A pulse liquid protein sequencer. The C-terminal amidation was determined by electrospray ionization mass spectrometry analysis.

Bioactivity of GWa (BACHEM), APGWa (SIGMA) and APGWa related peptides (EUROGENTEC)

Aliquots of synthetic peptides from 10^{-8} M to 10^{-4} M were tested on the oviduct bioassay in order to obtain qualitative and quantitative data about their activity.

Post-Translational Processing of GWa

Extract and incubation. Optic lobes just removed from anesthetised animal were homogenised in an ice cold Potter in saline phosphate buffer (KH_2PO_4 2.35 mM, K_2HPO_4 3.11 mM, NaCl 154 mM, pH 6.5) and centrifuged 2 min at +4°C. The supernatants were pooled and kept at −20°C until incubation. The assays were performed as described in Table 1. The incubation was stopped with 500 μl methanol-water-acetic acid: 90:9:1. Each tube was vortexed 5 s, centrifuged for 20 min at 12.000 *g,* and 500 μl of the supernatant was evaporated in a Speedvac concentrator.

HPLC Analysis. HPLC analysis was performed with a VARIAN-4050 integrator connected with one VARIAN-9012 solvent delivery system and with a Varian-9050 wave length UV-VIS detector set at 214 nm. All water and ACN solutions used in the HPLC analysis contained 0.1% TFA. The dry optic lobe extracts of each assay were resuspended in 150 μl of 0.1% TFA in water, injected in a 50μl injection loop and eluted by a 45 minute linear gradient of 1.33% of ACN per minute from 0 to 60% of ACN on a column Merck 100RP18 (4 × 250 mm, 5 μm).

Investigation of APGWa related peptides by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS). Six optic lobes kept in nitrogen liquid were heated at 100°C in 200 ml of 0.1N HCl for 5 min, homogeneised in a Potter, sonicated for 2 min and centrifuged 20 min at 12.000 *g*. The supernatants were pooled and loaded onto a C18 Sep-Pak. Bound material was eluted with 60% ACN/40% water in 0.1% TFA and evap-

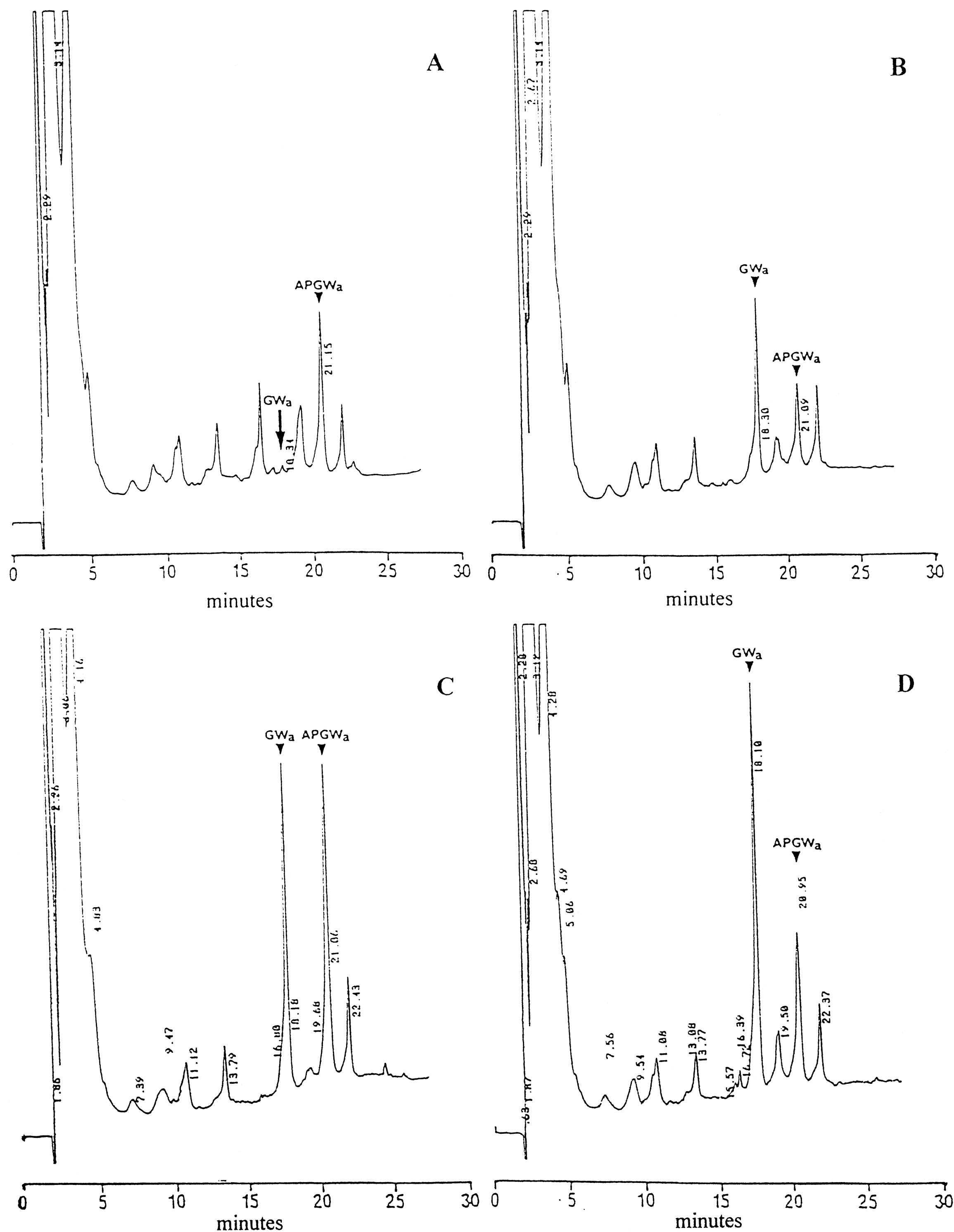

FIG. 3. Dipeptidylaminopeptidase activity in the optic lobe: HPLC analysis of the processing of synthetic APGWa into GWa. (A) APGWa + OL: no incubation. (B) APGWa + OL: one minute of incubation at 35°C. (C) APGWa + GWa + OL: no incubation. (D) APGWa + GWa + OL: one minute of incubation at 35°C.

orated in a Speedvac concentrator. The dry pellets were resuspended in 0.1% TFA in water and separated as described in the first step of purification of GWa. The fraction corresponding to RPGWa, KPGWa, TPGWa (1) and APGWa elution between 29 and 38% of ACN were collected, evaporated and analysed in MALDI-MS.

RESULTS

The 20 C18 fractionations were sufficiently reproducible to pool the fractions of the successive runs. An amount corresponding to 4 optic lobes (2 animal equivalents) was taken and tested in the oviduct bioassay. One active fraction, which induced a decrease of tonus, amplitude and frequency of the contractions of the oviduct was eluted at 12% acétonitrile as shown in Fig. 1a. The active fraction was successively eluted at 30% ACN (Fig. 1b), 18% ACN (Fig. 1c) and 10% ACN (Fig. 1d). HPLC analysis of the PTH-amino acids performed by the protein sequencer gave the following primary sequence: Gly-Trp. The determination of the molecular weight by mass spectrometry revealed a value of 259.9 Da and the occurrence of a C-terminal amidation. The complete structure of this new peptide is : Gly-Trp-NH_2. On the basis of the quantity of pure peptide (32 nmoles), we can estimate that each optic lobe at least contains 0.3 nmole peptide. Application of 10^{-8}M synthetic GWa inhibited the contraction for up to 16 minutes (Fig. 2). For this dose, the latency time was about 200 seconds and the amplitude of the main contractions was reduced at 30% of the basal amplitude. As shown by the Fig. 2, the threshold of activity was located between 10^{-9}M and 10^{-8}M. For APGWa, the threshold was near 3.10^{-5}M and for KPGWa, RPGWa and TPGWa the same threshold was observed: 10^{-5}M (Table 2). Therefore, the GWa appeared to be 3000 times more potent than APGWa and 1000 times more potent than KPGWa, RPGWa and TPGWa. Seasonal experiments showed that until the beginning of vitellogenesis, the oviduct was not receptive to synthetic GWa suggesting the absence of the receptors. The response of GWa appeared with the beginning of the biosynthesis of egg capsule material and yolk proteins in the genital apparatus. After incubation of 1 μg of synthetic APGWa with an optic lobe extract, the analysis of the incubation medium in HPLC revealed the processing of the tetrapeptide into new synthesised GWa suggesting the occurrence of a dipeptidylaminopeptidase activity (Fig. 3). The investigations to identify the tetrapeptide processed into GWa, i.e. purification by means of HPLC of the fractions coeluting with the presently known APGWa-related peptides (RPGWa, KPGWa, TPGWa (1) and APGWa) and analysis in MALDI-MS, did not allow the detection of any of them.

DISCUSSION

The primary sequence of the neuropeptide isolated and purified in this study is : Gly-Trp-NH_2. This peptide is the fifth member of the APGWa family and the first one identified in cephalopods. Although it appears as a truncated form, extractions performed in different conditions (liquid nitrogen organ powder in cold acetone or in cold methanol/water/acetic acid (90:9:1)) have shown that GWa was not an artefact. The quantity of pure peptide is so high (32 nmoles) that we should have easily detected the activity of the native form. Nevertheless, the occurrence of the dipeptide in optic lobes is likely related to the cleavage of one or few APGWa-related peptides. In many organisms, a class of aminopeptidases termed dipeptidylaminopeptidases (DPAP) remove N-terminal dipeptides (X-Ala, X-Pro) by hydrolyzing peptidyl bond following the alanine or the proline. The DPAP activity appears to be an alternative mechanism for the maturation of precursors onto bioactive proteins or peptides: peptides produced by amphibian skin, precursors of lytic peptides from honeybee venom, bactericidal peptides secreted in the insect haemolymph or extracellular proteases in yeasts (6). As well in *Sepia officinalis,* we have clearly demonstrated the ocurrence of a DPAP activity able to process synthetic APGWa into GWa. Nevertheless, the MALDI-MS analysis of optic lobe HPLC fractions corresponding to the retention times of the various XPGWa presently identified (APGWa, RPGWa, KPGWa and TPGWa) did not allow to detect any of them while this technique is sensitive enough to characterize the peptide content from even a single neuron (5).

Thus, the tetrapeptide processed into GWa could have a short half-life because of the high level of dipeptidylaminopeptidase activity in the optic lobes or/and this tetrapeptide could be a new XPGWa (excluding APGWa, RPGWa, KPGWa and TPGWa) for which the N-terminal aminoacid is yet unknown. With regard to the diversity and the distribution of this peptide family, i.e. GWa in cephalopods, APGWa in gastropod (7,13), no APGWa in the bivalve *Mytilus edulis,* but a precursor containing copies of RPGWa, KPGWa and TPGWa in the ratio of 5:1:1 (1), the occurrence of a new APGWa related peptide in cephalopods has to be considered. Experimental approaches based on molecular biology or immnulogical techniques should allow to elucidate this question. In gastropods the purification of APGWa performed from a muscle tissue of *Fusinus ferrugineus* (7) as well as the occurrence of APGWa in the penis complex of *Lymnaea stagnalis* (15) show that the tetrapeptide is the final product of the peptides expressed by APGWa precursor. In bivalves, the possible DPAP processing of KPGWa, RPGWa and TPGWa, products of the APGWa precursor of *Mytilus edulis,* has to be examined. Indeed, it is interesting to notice that the variations of APGWa precursor products only concerned the N-terminal amino acid and so allows the processing into GWa by a DPAP. Moreover, using synthetic APGWa-related peptides, Minakata et al (11) have previously shown on the ABRM (anterior byssus retractor muscle) of *Mytilus edulis* that GWa was the more potent APGWa-related peptides. This paper concluded that the dipeptide was the necessary and sufficient sequence to maintain biological activity and might be released as a neuromediator in some classes of molluscs. The activity of the final products of the APGWa precursor observed in the different classes of molluscs is very similar. In *Sepia officinalis,* GWa is clearly involved in the control of the oviducal motility and appears to be the most active APGWa-related peptide on the oviducal contractions. The mode of action, direct or indirect, is not fully elucidated. In the gastropod *Lymnaea stagnalis,* the APGWa is transported in the penis nerve to the male structure to control the mating behaviour inhibiting the contractions of the penis retractor muscles by the suppression of the excitatory effects of serotonin and dopamine (8). Nevertheless, RNA blot analysis from immature *Lymnaea stagnalis* reveals an early expression of the APGWa gene (13) suggesting functions other than the regulation of reproductive behavior.

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